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Development of a novel 3D gut-microbiota model for the assessment of absorption and metabolism of health supplements and therapeutic drugs

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A thesis submitted in partial fulfilment of the requirements of Keele University for the degree of Master of Philosophy

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Abstract:

The human body hosts a vast number of microorganisms such as bacteria, archaea, and viruses that are colonised inside and outside the body. This combination of microorganisms living within the human body is known as microbiota. The human microbiota varies depending on the anatomical site such as the skin, the respiratory tract, the gastrointestinal tract, and the genitourinary tract. Microbiota's composition and function can also vary according to the age, sex, race, and diet of its host.

This research focuses on the microbiota present in the gastrointestinal tract and how it can affect the absorption of molecules. By using two types of epithelial cell lines, Caco-2 and HT29-MTX and a bacterial strain, *lactobacillus* bacteria. To upgrade the existing Caco-2 model.

The cells were cultured in specialised plates until differentiation. Erythromycin, vitamin D3, and linoleic acid were used as test compounds. TEER and Lucifer yellow were used to monitor the integrity and formation of the monolayer. Absorption of the three molecules were studied by mass spectrometry.

The results indicated that the combination of cells and bacteria produced similar results to the ADMETox model currently in use the pharmaceutical industry.

Acknowledgment:

First and foremost, my deep gratefulness and indebtedness are to **Allah**: Master of worlds for helping me to complete this work.

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Many thanks go to my siters, brothers, and my friends for their support during my study.

Abbreviations:

Papp: apparent permeability coefficient

FBS: Foetal bovine serum

NEAA: Non-Essential Amino Acid

TEER: Transepithelial/transendothelial electrical resistance

DMSO: Dimethyl sulfoxide

Caco-2: Cancer coli-2

Immunoglobulin A: IgA

RFU: Relative fluorescence units

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Chapter 1: Introduction

1.1 Epithelial Cell Models

The intestinal epithelium cells have two major roles first, it acts as a physical barrier between the gastrointestinal lumen and the rest of the human body. Second, to ascertain the absorption of nutrients from the gut lumen to the rest of the body and the production of mucus that have protective anti-microbial properties which affect the composition of the microbiota (Lea, 2015b). The small intestinal epithelium is formed of several units of crypts and villi. The intestinal epithelium has four main types of cells that are derived from adult stem cells that are located at the crypt:

1- Absorptive cells.
2- Goblet cells which secrete mucus.
3- Enteroendocrine cells responsible for secretion of hormones.
4- Paneth cells that excrete antimicrobial peptides.

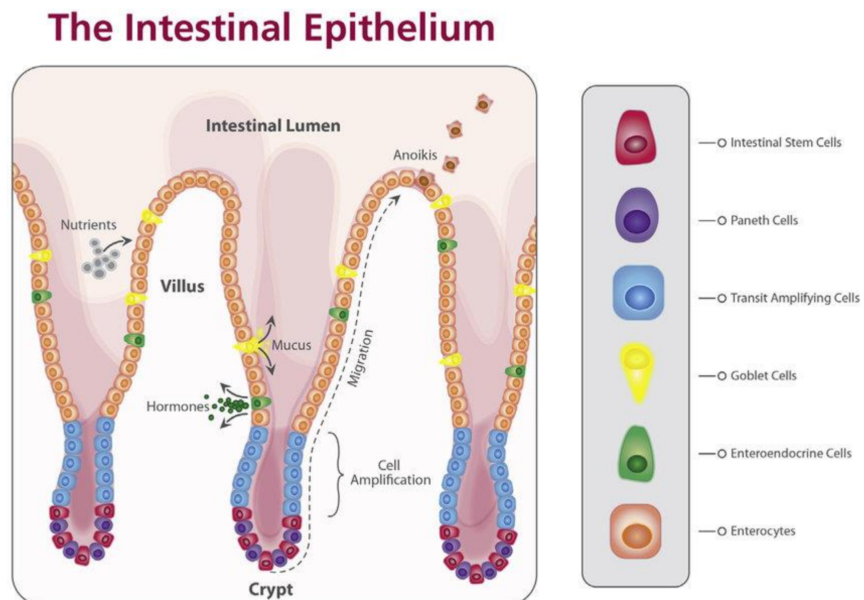

Figure 1: The different types of cells present in the intestinal lumen (Dey & Bradbury, 2018).

Microfold cells (M-cells) are found in the small intestinal epithelium overlaying with lymphoid tissue and Peyers patches (Lea, 2015b).

The single layer of columnar epithelial cells is not only acting as a mechanical barrier throughout the whole digestive tract, the epithelium is also coated with a viscous mucus layer that restricts the access of harmful microorganisms to the intestinal lumen by releasing antimicrobial proteins and neutralizing secretory Immunoglobulin A antibody (IgA) (Caruso, Lo, & Núñez, 2020; Lea, 2015b). The mucus covering the gut is composed of proteoglycans including mucin 2 (MUC2) that are secreted by the goblet cells (Caruso et al., 2020; Lea, 2015b).

The mucus in the small intestine is a single layer which lacks inner mucus but has many Paneth cells secreting antimicrobial peptides (Ayabe et al., 2000; Caruso et al., 2020). The large intestine has two layers of mucus, an outer one that has a large number of mucus-dwelling bacteria and an inner layer that contains a large number of microorganisms (Johansson, Larsson, & Hansson, 2011; Johansson et al., 2008; Lea, 2015b).

The epithelial membrane is selectively permeable to bacterial metabolites and nutrients through paracellular transport (Lea, 2015b). This form of transportation is regulated intercellular tight junctions (TJ), any disruption to the tight junction can trigger the mucosal immune system to react leading to inflammation (Lea, 2015b).

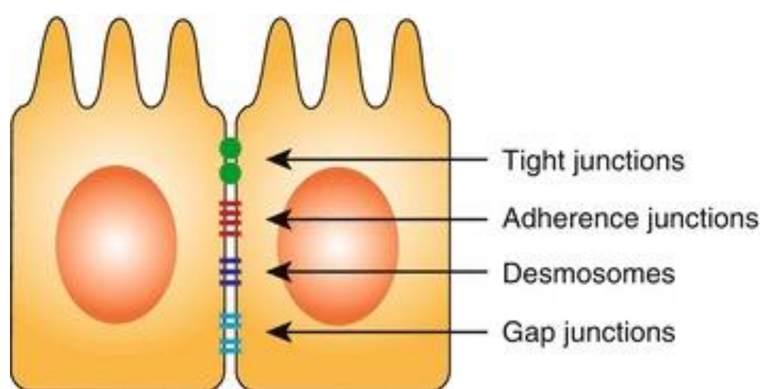

Figure 2: The figure illustrates a diagram of polarized epithelial cells, included types of intercellular contacts which are important components for communication between neighbouring cells and maintenance of barrier function (Lea, 2015b).

There are three different adhesive complexes connecting epithelial cells by: tight junctions, adherence junctions and desmosomes as shown in (Figure 2). These complexes have transmembrane proteins which interact with adaptor proteins as intracellular and adjacent cells that link to cytoskeleton. Paracellular permeability increases and decreases with

the TJ barrier if any disruption happens to the intestinal TJ (Lee, 2015). Permeation of luminal proinflammatory molecules have the ability to induce activation of the mucosal immune system. This can lead to tissue damage and inflammation, with TJ barrier reduction having significant impact on both whole body health and gut health (Lea, 2015b).

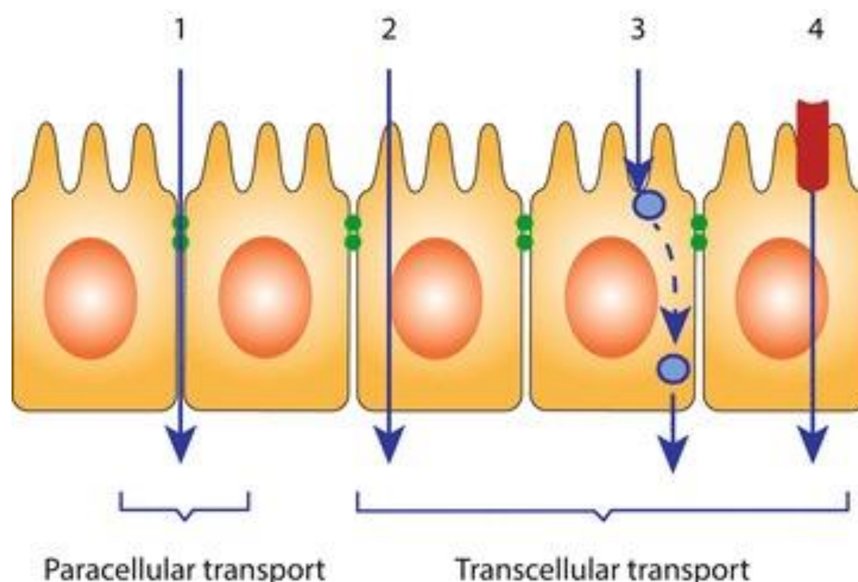

Figure 3: The figure illustrates different transport and modes of absorption with intestinal epithelium such as paracellular transport, vesicle-mediated transcytosis and passive diffusion of molecules from the apical to the basolateral side (Lea, 2015b).

There are four transport mechanisms which allow macromolecules to be transported through the epithelial cell layer as well as selective uptake (Figure 3):

1- Paracellular transport.

2- Passive diffusion.

3- Vesicle-mediated transcytosis.

4- carrier-mediated uptake.

1.2 Microbiota

The environment plays an important role in the way humans have evolved (Cong & Zhang, 2018). The human body hosts a large number of microorganisms such as bacteria, archaea, and viruses that are colonised inside and outside the body (Ursell et al., 2014). While the microorganisms living within the human body is known as microbiota, the microbiome is

"the collective genomes of our microbial symbionts" (Turnbaugh et al., 2007). The diversity of microbiota was noted first by Antonie van Leewenhoek in the early 1680s (Ursell, Metcalf, Parfrey, & Knight, 2012). Antonie van Leewenhoek compared his oral to faecal microbiota, noticing the differences between the microbes in these two inhabits (Ursell et al., 2012). Until recently, studies of microbiology have been focusing on the microorganisms that cause diseases, rather than studying the benefits of these microorganisms within the human body (Peterson et al., 2009).

The human microbiome now is considered a whole organ (Eckburg et al., 2005) with the estimated number of microbiota cells as one hundred trillion (Ursell et al., 2012) hence, it is considered as "a second genome" (Grice & Segre, 2012). The human microbiota varies depending on the location such as the skin, the respiratory tract, the gastrointestinal tract, and the genitourinary tract (Willey, 2015). The microbiota is not a static equilibrium (Willey, 2015), it colonises the intestine shortly after birth and changes as the human ages (Rogier et al., 2014). Infants acquire most of their microbiota from their mothers, if they are born vaginally (Rogier et al., 2014). When babies are born by caesarean they acquire their microbiota from the surrounding environment typically from the one found on the human skin (Dominguez-Bello et al., 2010).

Microbiota composition and function can also vary with age, sex and race (Figure 4) (Hollister, Gao, & Versalovic, 2014). Genetic diseases, diets, environmental factors, drug therapies, recreational drugs, stress, infections, smoking, drinking and more can have a negative impact on the microbiota as well (Aron-Wisnewsky, Doré, & Clement, 2012; Gibson & Roberfroid, 1995; Guinane & Cotter, 2013; Sartor, 2012; Sommer & Bäckhed, 2013).

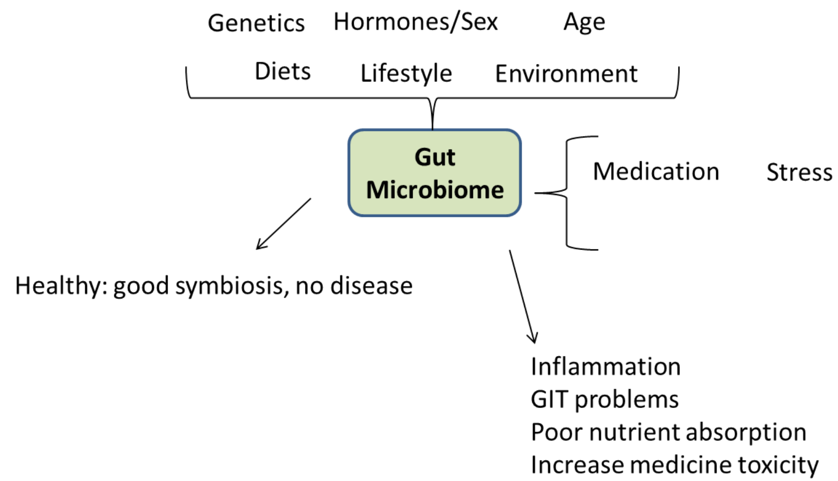

Figure 4: Holistic interaction of the gut microbiome in healthy and disease stages

Microbiota have proven to be extremely important for human health but studies using germ-free animals have shown they are not indispensable, when working under restricted laboratory conditions. Nevertheless, free-germ animals still must be provided with essential nutrients. Interestingly, anatomical differences have been found in these animals, for example the enteric nervous system in free-germ animals is not fully developed, and the blood brain barrier is more permeable. Serotonin levels in germ-free animals are also low (Winek, Dirnagl, & Meisel, 2016). Research points in the direction implying microbiota contribute to the development of the immune system and the onset of different conditions.

1.3 Microbiota Development

In the recent decades, a high volume of research has suggested that microbiota has an impact on our health via number of mechanisms (Wang, Yao, Lv, Ling, & Li, 2017). This co-existence of bacteria and gut epithelial is paramount for human health, allowing the absorption of nutrients as well as modulating different signals, (Figure 5) (Faderl, Noti, Corazza, & Mueller, 2015; Shen & Wong, 2016).

There are around 3 million bacteria in the human gut comprising of around 1000 different species of bacteria (Franks, 2012; Gorbach., 1995; Sears, 2005). These species include *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria* families (Sears, 2005;

Tidjani Alou, Lagier, & Raoult, 2016). From the enterotypes, three take central stage, *Prevotella*, *Ruminococcus* and *Bacteroides* (Dinan & Cryan, 2017).

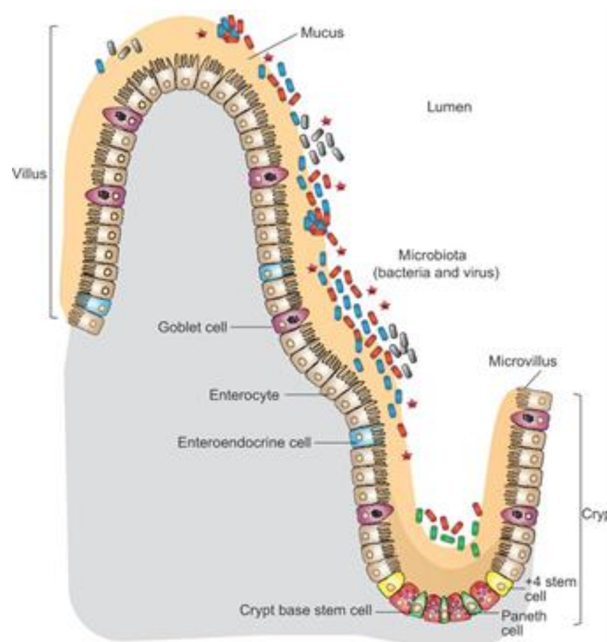

Figure 5: Bacterial-gut epithelial interactions (Wong, Vanhove, & Watnick, 2016).

In healthy individuals, where there is homeostasis in physiological processes, the micro-organisms residing in the gut microbiota are commensal. Though, there is discrepancy in the bacterial composition in the human gut, it is consensus from a qualitative point of view, that in the stomach *Helicobacter pylori* can be found, while *Lactics*, *Enterics*, *Enterococci* and *Bifidobacteria* are present in the small intestine and *Bacteroides*, *Lactics*, *Enterics*, *Enterococci*, *Clostridia* and *Methanogens* are present in the colon (David et al., 2014; Todar, 2012). These genera can be divided into anaerobic (not requiring oxygen to survive) genera in where we can find *Bifidobacterium*, *Clostridum*, *Bacteroides* and *Eubacterium* and aerobic (depending on oxygen to survive) genera in where we can find *Escherichia*, *Enterococcus*, *Strpetococcus* and *Klebsiella* (O'Hara & Shanahan, 2006). In the duodenum metabolic activities include digestion of proteins, monosaccharides, short-chain fatty acids (SCFAs) and immunomodulation. In the jejunum there is absorption of free fatty acids, calcium, and vitamins A, D, E and K. In the ileum, there is absorption of vitamin B12 and bile acids. From duodenum to ileum, the main bacteria present are *Firmicutes* and *Protobacteria.* In the colon,

there is absorption of water and SCFAs with the bacteria preponderant being *Bacteroidetes* and *Firmicutes* species (Scheithauer, Dallinga-Thie, de Vos, Nieuwdorp, & van Raalte, 2016).

A study described that vegetarian subjects were found to have more *Firmicutes* over *Clostridia* and *Bacteroidetes*, while non-vegetarians had more *Bacteroidetes* over *Firmicutes* and *Clostridia,* being similar to inflammatory bowel disease (IBD) sufferers, though IBD patients had a higher bacterial count of the latter two (Bamola et al., 2017). World location also plays an important role as it determines the types of diet. Looking at the faecal microbiological composition, in Burkina Faso (BF) African children with a diet based mainly on cereals, legumes and vegetables had 57% of *Bacteroidetes* and 27% of *Firmicutes* while European diets are high in animal protein, sugar, starch and fat but low in fibre. Children a diet high in animal protein, sugar, starch and fat while low in fibre children had 22% and 63% respectively.

The BF African children also had *Prevotella*, *Xylanibacter* and *Treponema* which would be an indication of high fibre intake and nutrient maximisation. Other factors also played a role such as age of breast-feeding, variety of vegetables and animal protein as well as sanitation, access to antibiotics and climate (De Filippo et al., 2010; F. Thomas, Hehemann, Rebuffet, Czjzek, & Michel, 2011). Some studies suggest *Bacteroides* comprise around 27.8% of the human gut with *Firmictures* comprising 38.8%, *Proteobacteria* 2.1% and *Actinobacteria* 8.2% with about to 1000 species (D'Argenio & Salvatore, 2015; Qin et al., 2010).

1.4 Gut microbiota and Metabolism

The human microbiota provides humans with unique and specific enzymes and biochemical pathways, which are beneficial to the host (Gill et al., 2006). This includes, the metabolism of undigested carbohydrates and the biosynthesis of vitamins (Roberfroid, Bornet, Bouley, & Cummings, 1995). Microbiota also provide a physical shield to protect the host from harmful pathogens by competitive exclusion and the production of antimicrobial

substances (Cash, Whitham, Behrendt, & Hooper, 2006; Hooper, Stappenbeck, Hong, & Gordon, 2003; Schauber et al., 2003). Microbiota seem, as well, to be essential for the development of intestinal mucosa and the immune system of the host (Wang et al., 2017).

The relationship between gut and bacterial enzymes provides a source of energy and nutrients to both the host and the bacteria. One of the main chemical roles of bacterial enzymes is to ferment non-digestible elements of the human diet. The main substrates for the fermentation process belong to the carbohydrate family including large polysaccharides (cellulose, pectin, and starch), oligosaccharides, alcohols and sugars. Proteolytic fermentation is also possible, proteins, peptides and glycoproteins can be fermented to smaller molecules (Vattem & Maitin, 2015).

Several studies have identified metabolites that have been correlated to human health and diseases. Some of these metabolites include:

- 10-hydroxy-cis-12-octadecenoate (HYA) by *Lactobacillus plantarum*.
- Short-chain fatty acids (SCFAs, including acetate, propionate,butyrate, isobutyrate, 2-methylpropionate,valerate, isovalerate, hexanoate) and long-chain fatty acid metabolites such as conjugated linoleic acid (CLA) by *Lachnospiraceae*, *Lactobacillus*, *Bifidobacteria*, *Faecalibacterium prausnitzii*, *Propionibacterium*, *Firmicutes,* *Propionibacterium*, *Bacteroides*, *Negativicutes*, *Selenomonas ruminantium*, *Roseburia inulinivorans*, *Escherichia coli.*
- Trimethylamine (TMA) and trimethylamine N-oxide (TMAO) by *Desulfovibrio*, *Proteus mirabilis*, *Ruminococcus*, *Akkermansia muciniphilia*;
- Tryptophan metabolites (indole-3-propionate (IPA), indole sulfate, indole-3-aldehyde and indoxyl-sulfate (IndsS)) by *Lactobacillus*, *Bifidobacterium longum*, *Bacteroides fragilis*, *Parabacteroides distasonis*, *Clostridium bartlettii*, *E. hallii*, *Clostridium sporogenes*.
- Tyrosine, phenylalanine metabolites and phenyl derivatives (benzoic acid, hippuric acid, 2-hydroxyhippuric acid, 2-hydroxybenzoic acid, 3-hydroxyhippuric acid, 3-

hydroxybenzoic acid, 4-hydroxybenzoic acid, 3-hydroxyphenylpropionate, 4-hydroxyphenylpropionate, 3-hydroxycinnamate, 4-methylphenol, tyrosine, phenylalanine, 4-cresol, 4-cresyl sulfate, 4-cresyl, glucuronide, 4-hydroxyphenylacetate, 3,4-dihydroxyphenylacetate, phenylacetylglycine, phenylacetylglutamine, phenylacetylglycine, phenylacetate, phenylpropionate, phenylpropionylglycine, cinnamoylglycine) by *Clostridium difficile, F. prausnitzii*, *Bifidobacterium*, *Subdoligranulum* and *Lactobacillus*.

- Bile derivatives (cholate, hyocholate, deoxycholate, chenodeoxycholate, α-muricholate, β-muricholate, Ω-muricholate, taurocholate, glycocholate, taurochenoxycholate, glycochenodeoxycholate, taurocholate, tauro-a-muricholate, tauro-b-muricholate, lithocholate, ursodeoxycholate, hyodeoxycholate, glycodeoxylcholate, taurohyocholate, taurodeoxylcholate) by *Lactobacillus*, *Bifidobacteria*, *Enterobacter*, *Bacteroides*, and *Clostridium.*
- Choline derivatives (methylamine, dimethylamine, trimethylamine, trimethylamine-N-oxide, dimethylglycine, betaine) by *Faecalibacterium prausnitzii* and *Bifidobacterium*
- Vitamins (vitamin K, vitamin B12, biotin, folate, thiamine, riboflavin, pyridoxine) by *Bifidobacterium.*
- Polyamines (putrescine, cadaverine, spermidine, spermine) by *Campylobacter jejuni* and *Clostridium saccharolyticum*
- Lipids (conjugated fatty acids, LPS, peptidoglycan, acylglycerols, sphingomyelin, cholesterol, phosphatidylcholines, phosphoethanolamines, triglycerides) by *Bifidobacterium*, *Roseburia*, *Lactobacillus*, *Klebsiella*, *Enterobacter*, *Citrobacter* and *Clostridium.*
- Other molecules (D-lactate, formate, methanol, ethanol, succinate, lysine, glucose, urea, a-ketoisovalerate, creatine, creatinine, endocannabinoids, 2-arachidonoylglycerol, (2-AG), N-arachidonoylethanolamide) by *Bacteroides*,

Pseudobutyrivibrio, *Ruminococcus*, *Faecalibacterium*, *Subdoligranulum*, *Bifidobacterium*, *Atopobium*, *Firmicutes* and *Lactobacillus.*

These metabolites have a range of roles with different activities such as anti-cancer, anti-inflammatory, effects on energy expenditure, intestinal barrier function, insulin sensitivity, kidney function, atherosclerosis, mucosal equilibrium, signalling, anti-oxidant and more (Holers et al., 2018; Nicholson et al., 2012; Noah, Donahue, & Shroyer, 2011).

Short-chain fatty acids can be produced by *Lactobacillus* and *Bifidobacterium* species and used by the human cells for signalling or substrates (Tan et al., 2014). Norepinephrine and dopamine can be produced by *Escherichia*, *Bacillus* and *Saccharomyces* species (Alkasir, Li, Li, Jin, & Zhu, 2017). Butyrate regulates the intestinal functions including its motility, production of mucus, visceral sensitivity, the epithelial barrier, immune homeostasis and the mucosal oxygen gradient (Kelly et al., 2015). The gut microbiota produces the vitamins K, B9, B12, and B2 (Bailey & Gregory, 1999; Barile et al., 2013; DiNicolantonio, Bhutani, & Keefe, 2015; Schramm et al., 2014; Spence, 2016; Woo, Kwok, & Celermajer, 2014).

1.5 Gut microbiota and Diseases

The gut epithelium is a complex setting of goblets and ciliated intestinal cells anchored onto epithelial cells and filled with transporters, metabolising enzymes and channels (Abreu, 2010; Gallo & Hooper, 2012; L. W. Peterson & Artis, 2014).

Goblet cells in the gut epithelium secret a mucus which acts as a lubricant and protects the epithelial cell layer against irritation from mucosal contents, and Paneth cells in the crypts of the small intestine produce peptides with bactericidal properties (Allen & Flemström, 2005). The combination of epithelial cells and mucus form the mucosal barrier. Several components of the immune system are present in the mucosal barrier, including antimicrobial peptides, IgA antibodies, macrophages, dendritic cells regulatory T-cells, helper t-cells and neutrophils, (Figure 6). Antigens are also monitored by CD4+ and CD8+ T-cells as well as he

Tumour Necrosis factor (TNF) family (Allaire et al., 2018; Allen & Flemström, 2005; Maldonado-Contreras & McCormick, 2011; Pott & Hornef, 2012).

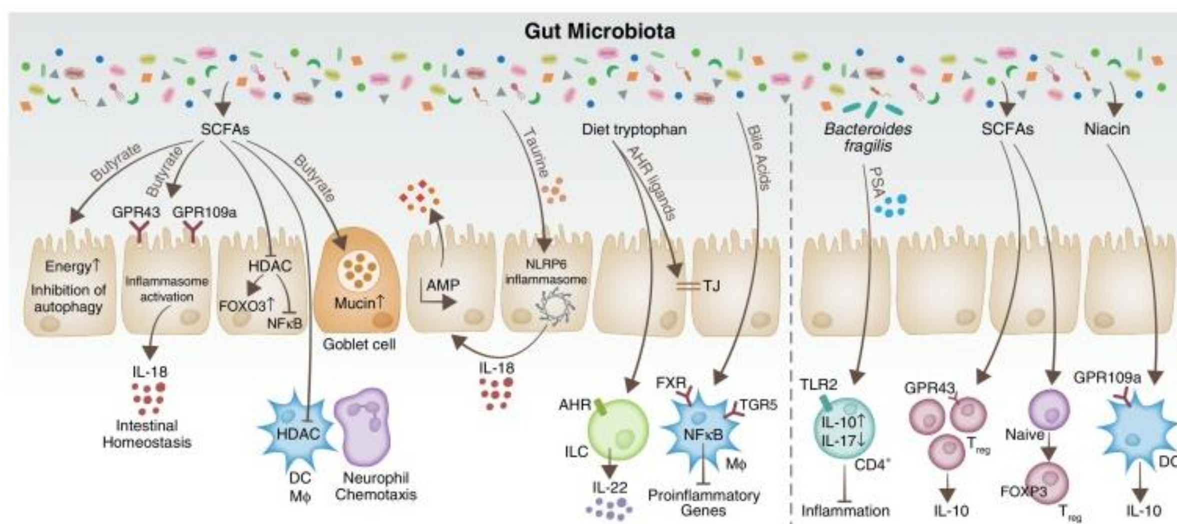

Figure 6: Modulation of immune signalling through microbial metabolites. Short-chain fatty acids (SCFAs), N-oxide (TMAO), 4-ethylphenylsulfate (4-EPS) (Levy, Blacher, & Elinav, 2017).

Alteration of the equilibrium of the gut microbiota has implications in the development of diseases. It has been documented that an increase in the ratio of *Firmicutes* to *Bacteroidetes* has a role on irritable bowel syndrome, while a reduced ratio of *Bacteroidetes* to *Firmicutes* has roles on obesity (Tidjani Alou et al., 2016). *Firmicutes* through their metabolic activities have roles in the inhibition of pathogen growth, cholesterol synthesis, and insulin resistance (Nicholson et al., 2012). Increased presence of *Clostridium* species, *Ruminococcus torques* and *E. coli* are responsible for some features of Crohn's disease. Colon cancer seems to have an abundance of *Fusobacteria* and *Coriobacteria* while rheumatoid arthritis has less *Bifidobacteria* and lower count of *Bacteroides*-*Porphyromonas*-*Prevotella* (Nicholson et al., 2012; Paul et al., 2015). *Lactobacillus* and *Bifidobacterium* assist with the regulation and absorption of lipids, detoxification of xenobiotics and strengthening of the immune function (Nicholson et al., 2012).

1.5.1 Obesity and Cardiovascular risk

Obesity rate has increased globally (Reilly, El-Hamdouchi, Diouf, Monyeki, & Somda, 2018) and its association with health problems such as diabetes type 2 and dyslipidemia, impacts cardiovascular health and metabolic diseases (Schiattarella, Sannino, Esposito, & Perrino, 2019). Studies on germ- free mice and conventional mice that have been fed with the same high fat diet (HFD) showed a reduction in obesity and weight gain in the first group but when they received a faecal transplantation from conventional HFD fed mice they restored their capability to gain weight (Peter et al., 2006; Ridaura et al., 2013; Turnbaugh, Bäckhed, Fulton, & Gordon, 2008). The sharing of gut flora between the two subjects has showed transmission of metabolic alterations to the germ-free mice and their ability to replicate the metabolic phenotype (Ridaura et al., 2013). The ratio of *Firmicutes* and *Bacteroidetes* increased in both mice and human who are obese (Ley et al., 2005; Ley, Turnbaugh, Klein, & Gordon, 2006), changes occurred not only in gut bacterial composition but also in the phylum levels. Hydrogen-producing bacteria *Prevotellaceae,* from the *Bacteroidetes* phylum level, was found to be high in the obese subjects compared to thinner individuals (Schiattarella et al., 2019). It appears that alterations of polysaccharides fermentative bacteria effect host intestine absorption in addition to appetite dysregulation (Chakraborti, 2015). When dietary intervention is applied, this ratio of *Firmicutes* and *Bacteroidetes* is reduced (Karlsson et al., 2012; Qin et al., 2012; Turnbaugh et al., 2009; Turnbaugh et al., 2006).

Intestinal epithelial barrier permeability might be affected by the changes of the microbial flora that alter glucose sensitivity and absorption which promotes insulin resistance (Tanti, Ceppo, Jager, & Berthou, 2013). The abundance of some butyrate-producing bacteria seems to be reduced in diabetes type 2 (Qin et al., 2012). When patients have a high pro-inflammatory state, as well as presenting low insulin sensitivity and metabolic syndrome, and

are treated with vancomycin, reduction of SCFAs in the gut butyrate is detected (Vrieze et al., 2014). However, when faecal transplantation was done from slimmer individuals to these patients, they have shown improvements in their insulin sensitivity and the abundance of their butyrate-producing bacteria was increased (Kimura et al., 2013; Maslowski et al., 2009; Samuel et al., 2008; Tolhurst et al., 2012).

The gut microbiota appears to be responsible for low grade inflammation, insulin resistance and increase cardiovascular risk. The culprit seems to be the production of lipopolysaccharides (LPSs), these endotoxins are produced in the wall of gram-negative bacteria. A diet rich in fat appears to increase the permeability of LPS in the intestinal barrier (Bottazzo, Manco, & Putignani, 2010). LPS promotes the secretion of pro-inflammatory cytokines through binding to Toll-like receptors (TLRs) and activating the TLR4/CD14 complex, towards pro-inflammatory pathways involving TNF-α, IL-1 and IL-6 as well as reactive oxygen species ROS generation (Gibbs, Del Vecchio, & Shaffer, 1992; Kristine, Trine, & Oluf, 2015). LPS are then transported as chylomicrons to the bile for clearance (Bottazzo et al., 2010). A study on rats suggested that administration of *Bifidobacteria* helped reduce the levels of LPS (Z. Wang et al., 2006).

In 1999, the Bruneck study, demonstrated association between LPSs and cardiovascular risk, the endpoints were early atherosclerosis in the carotid arteries, determined by high-resolution duplex ultrasound and incident cardiovascular disease. The levels of LPS ranged from 6.0 to 209 pg/ml and at 50 pg/ml the patient faced a 3-fold risk of incident of atherosclerosis. A more recent study, the Wandsworth Heart and Stroke Study linked the risk factors for cardiovascular disease with ethnicity (Miller et al., 2009; Wiedermann et al., 1999).

Preclinical and clinical studies have indicated dysbiosis in gut microbiota population and hypertensive heart disease (Mell et al., 2015; Qi, Aranda, Rodriguez, Raizada, & Pepine, 2015; Yang et al., 2015). Animal studies of hypertension have documented the role of gut microbiota in blood pressure (Schiattarella et al., 2019) as Dhal salt sensitive rats,

spontaneously hypertensive rats, chronic infusion of Angiotensin II mice, and rodent models showed reduction in abundance and diversity in the microbiota composition (Adnan et al., 2016; Mell et al., 2015; Yang et al., 2015).

In a recent study, bacterial DNA was found in the vessel wall at the site of atherosclerotic plaques in a patient, and a correlation between these bacteria and gut microbiota was detected (Koren et al., 2011; Ott et al., 2006). Some studies have suggested that the presence of bacteria in the plaques may have a role in the instability and development of ischemic heart disease between patients who have stable atherosclerotic plaques versus patients with unstable atherosclerotic plaques by using metagenomic sequencing (Schiattarella et al., 2019). In the latter group low abundance of faecal butyrate produced by *Roseburia* genus was present, this noted changes in the gut microbiota metabolic activity in the pro-inflammatory state of patients who have unstable atherosclerotic plaques (Karlsson et al., 2012; Maslowski et al., 2009).

1.5.2 Rheumatoid Arthritis

Rheumatoid arthritis (RA) affects around 1% of the population world-wide. It is an autoimmune disease-causing joint pain, stiffness, and swelling (NHS, 2016). The main factor causing rheumatoid arthritis is the immunologic dysregulation that leads to inflammation with the presence of autoreactive T and B cells (Gregersen, Silver, & Winchester, 1987).

Recent studies suggesting that pro-inflammatory molecules that are produced by bacterial products can compromise the intestinal barrier integrity (Taneja, 2014). In a study, fatty acids produced by gastrointestinal bacteria were analysed and showed rheumatoid arthritis patients had different microbiota (Eerola et al., 1994). In another study, faecal microbiota from people suffering rheumatoid arthritis (RA) and non-inflammatory fibromyalgia was compared with the RA patients having fewer amounts of *Bifidobacterium*, bacteria of the *Bacteroides–Porphyromonas–Prevotella* group and the *Eubacteriumrectale–Clostridium coccoides* (Taneja, 2014).

This suggests that chronic inflammatory processes in Rheumatoid Arthritis could be promoted by overgrowth in pathogenic bacteria or immune-modulating bacterial populations being lowered.

1.5.3 Dementia

The influence of the gut microbiota has greater implications in dementia conditions, as it has been found that early inflammatory signals between the gut and the brain can contribute on the development of dementia, (Figure 7) (Alkasir et al., 2017).

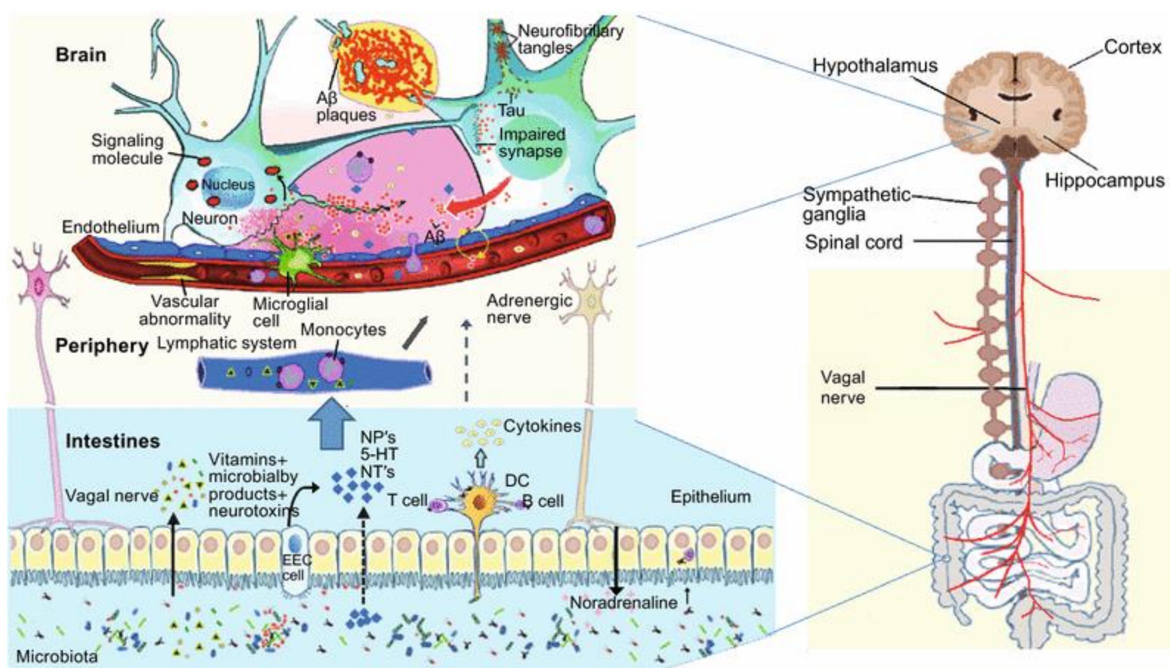

Figure 7: Key signals in the development of dementia. The gut microbiota regulation of neuro-inflammation and the hypothalamic–pituitary–adrenal (HPA) axis activity and may lead to AD. Bacterial products communicate with the brain through the bloodstream and the area postrema, via cytokine release from mucosal immune cells. NP: Neuropeptide; NT: Neurotransmitter; 5-HT: 5-hydroxytryptamine; DC: Dendritic cell; EEC: Enteroendocrine cell; Aβ: amyloid beta protein; AD: Alzheimer's disease (Alkasir et al., 2017).

The microbiota supports the brain's function in a number of ways, including the regulation of the hypothalamic-pituitary-adrenal (HPA) axis, which in turn releases cortisol and cytokines as well as several neurotransmitters, neuropeptides, endocrine hormones and immune-modulators (Alkasir et al., 2017).

Commensal and pathogenic bacteria were found to be essential in the brain-gut axis interaction (Alkasir et al., 2017). Bacteria has been responsible for producing or releasing of neuropeptides; gamma-aminobutyric acid (GABA) is a metabolic product from *Lactobacillus*

and *Bifidobacterium*; serotonin can be produced by *Streptococcus*, *Escherichia*, *Enterococci*, *Enterococcus*, *Lactococcus*, and *Lactobacillus*; norephinefrine can be produced by *Bacillus*; Acetylcholine is a product by *Lactobacillus* and *Bacillus*, histamine can be the result of metabolism by *Lactobacillus*, *Lactococccus*, *Streptococcus* and *Enterococcus*; dopamine can be the result of reactions from *Escherichia*, *Bacillus*, *Lactococcus, Lacotbacillus*, and *Streptococcus* (Alkasir et al., 2017; Ghaisas, Maher, & Kanthasamy, 2016).

1.6 Models to study Gut microbiota

1.6.1 Animal models

Germ-free (GF) rodents breeding started in the 1930s and since 1960s germ-free rodents been standard starting point to produce barrier-bred laboratory animals with a specific pathogen free (SPF) (Hansen & Nielsen, 2014; Kirk, 2012). Furthermore, germ-free rodents are important to study the effects of microbial mono- and polycolonisations on the host phenotype (Bercik et al., 2011; Peter et al., 2006; Wen et al., 2008). Studying the interaction between host-microbiome and its dysbiosis implication on the human health have gain massive interest and has been the subject of several studies (Qin et al., 2012; Salonen, de Vos, & Palva, 2010), and the use of germ-free rodents to transplant microbiotas have been the subject of recent investigations (Lundberg, Toft, August, Hansen, & Hansen, 2016).

1.6.2 *In vitro* models

One of the main advantages of using *in vitro* models is the controlled environment (von Martels et al., 2017). Frequently, intestinal cell lines such as Caco-2, HT-29, T-84 and DLD-1 originated from gastrointestinal tumours and are used to represent the human gastrointestinal epithelium. Even though, the epithelial characteristics of these cell lines might be compromised because of the originally of the cells, they can be used in other experiments such as Ussing chamber experiments to evaluate the transportation of molecules permeability through the epithelial cell layer. As some of these cell lines have an advantage due to the intactness of the intestinal mucosa layer (Browning & Trier, 1969; Tsilingiri et al., 2012).

In some studies of drug metabolism, *ex vivo* models such as precision-cut intestinal tissue slices (PCIS) are used (de Kanter et al., 2005; M. Li, de Graaf, & Groothuis, 2016). Recently, intestinal organoids are being used as models of the human intestinal epithelium that contain all main types of epithelial cells such as enterocytes, goblet cells, enteroendocrine cells and Paneth cells (Lukovac et al., 2014). These models can be grown *in vitro* from resident stem cells in the gut and remain genetically stable in culture for many cell divisions (Lukovac et al., 2014).

Investigating the microorganisms of the microbiota has been done using several techniques from molecular epidemiology, microbial ecology, and microbiology (Bäckhed et al., 2012; Garrett, 2015; Hsiao et al., 2013; Petersen & Round, 2014; Trompette et al., 2014). However, most of studies have been focusing on the bacteria that are based in the lumen of the colon (Sarangi, Goel, & Aggarwal, 2019).

The pharmaceutical industry, more precisely the pre-clinical area, makes good use of a simplistic intestinal model grown on filters using Caco-2 cell line, this model has been the workhorse approved by the FDA to study transporters inhibition, metabolism, and permeability, with the main feature being reproducibility and low cost. This model lacks many features of the human gut.

Recent approaches using genomic analyses, like metagenomic species, study the different species in the gut but do not provide information on how drugs and nutrients are absorbed. Chip technologies have produced the gut-on-chip model (Elveflow), HuMiX and organoids (Bein et al., 2018).

1.6.2.1 Caco-2 cell line

In the 1970s different types of epithelial cell lines were established from gastrointestinal tumours (Lea, 2015a). The main goal of this was to study the different mechanisms of cancer development and cytostatic therapies (Lea, 2015a; Sambuy et al., 2005). One cell line **Ca**ncer ***co****li*-2 (Caco-2) had unique characteristics. It differentiated spontaneously when reaching confluence (Lea, 2015a). It was first established by Jorgen Fogh

at the Sloan-Kettering Cancer Research Institute from a human colorectal adenocarcinoma (Fogh, Wright, & Loveless, 1977; Lea, 2015a).

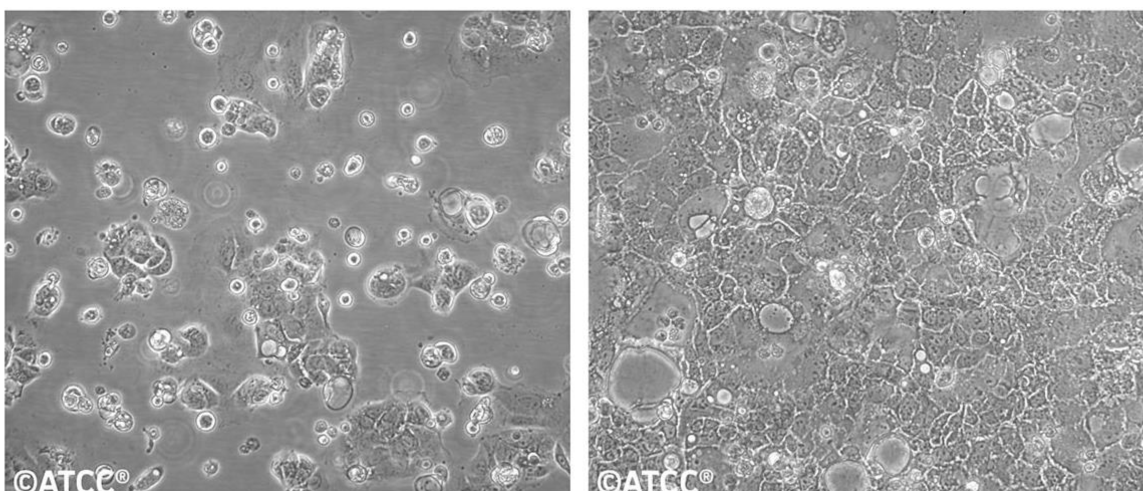

Figure 8: Caco-2 cells in culture at low and high density (ATCC, 2016).

1.6.2.1.1 Features and Mechanisms

A study by Engle *et al* (1998) showed that differentiated Caco-2 cells look-like the small intestinal morphologically and functionally (Engle, Goetz, & Alpers, 1998). As Caco-2 cells have the morphology and function of intestinal epithelial cells the membrane they have been divided into two major regions:

- Apical membrane is the top surface of the cell that faces the outside of the organism (Lodish, 2016).
- Basolateral membrane is the bottom surface of the cell that faces the inside of the organism or the bloodstream side (Lodish, 2016).

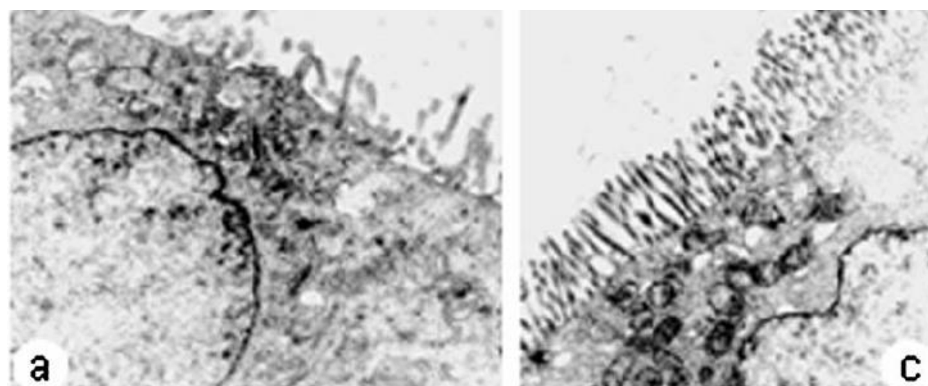

Figure 9: differentiated Caco-2 cells microvilli's at early phase of culture comparing at confluence (Simon-Assmann, Turck, Sidhoum-Jenny, Gradwohl, & Kedinger, 2007).

Caco-2 cells grow in culture as adherent monolayers and when reaching confluence, they polarize to acquire apical brush border characteristics with microvilli and occluding junctions (tight junctions) are formed between adjacent cells (figure 9) (Lea, 2015a).

Table 1: Properties of Caco-2 cell lines (Lea, 2015a).

Growth	Grows in culture as an adherent monolayer of epithelial cells
Differentiation	Takes 14–21 days after confluence under standard culture conditions
Cell morphology	Polarized cells with tight junctions and brush border at the apical side
Electrical parameters	High electrical resistance
Digestive enzymes	Expresses typical digestive enzymes, membrane peptidases and disaccharidases of the small intestine (lactase, aminopeptidase N, sucrase-isomaltase and dipeptidylpeptidase IV)
Active transport	Amino acids, sugars, vitamins, hormones
Membrane ionic transport	Na+/K+ ATPase, H+/K+ ATPase, Na+/H+ exchange, Na+/K+/Cl− cotransport, apical Cl− channels
Membrane non-ionic transporters	Permeability glycoprotein (P-gp, multidrug resistance protein), multidrug resistance-associated protein, lung cancer-associated resistance protein
Receptors	Vitamin B12, vitamin D3, EGFR (epidermal growth factor receptor), sugar transporters (GLUT1, GLUT2, GLUT3, GLUT5, SGLT1)
Cytokine production	IL-6, IL-8, TNFα, TGF-β1, thymic stromal lymphopoietin (TSLP), IL-15

Since Caco-2 cells have been established, they have been popular in laboratories worldwide (Per Artursson, Palm, & Luthman, 2001; Lea, 2015a). It is been reported that variables such as transepithelial electrical resistance (TEER) and proliferation rate are increased with increasing passage number (Briske-Anderson, Finley, & Newman, 1997). The TEER measurements will be affected by the late passage of the cells as they start to grow in multilayers (Lea, 2015a).

1.6.2.1.2 Relevance to Human *In Vivo* Situation:

In order to mimic the conditions in the intestine,Caco-2 cells need to be cultured in specialised culture plates which have permeable filter inserts, which can be purchased from a number of manufacturers such as Becton Dickenson, Corning, Costar, etc (Lea, 2015a).

Figure 10: : The figure illustrates the different plates and filters to culture cells (Sigmaaldrich, 2021)

Culturing on filters improves the morphology and function of the cells (Lea, 2015). There are number of studies that found polarised Caco-2 monolayers is a reliable correlative to study the absorption of oral drugs (Lea, 2015). A vast number of studies have found a high correlation between absorption and Caco-2 permeability coefficients (P. Artursson & Karlsson, 1991; Cheng, Li, & Uss, 2008; Sun, Chow, Liu, Du, & Pang, 2008).

A large number of enzymes and transporter proteins produced in normal human intestinal epithelium are expressed by Caco-2 cells (Bourgine et al., 2012). Gene expression profiles variations are only noted in the mucosal epithelium of the gastrointestinal tract, but it can be observed in crypt-villus axis (Anderle et al., 2005).

1.6.2.1.3 Applications of Caco-2

Culturing Caco-2 can provide information about the biological and biochemical basis of barrier properties of the intestinal mucosa (Lea, 2015a). Also, it could unravel valuable information about the absorption of drugs and dietary components relevant for both the pharmaceutical and the food industry (Lea, 2015a). Therefore, Caco-2 cell line has been used in different range of application such as:

- Studying mechanisms and effects of microbiota on the barrier function of the intestinal epithelium (Shimizu, 2010).
- Explanation of drugs transportation pathways and food components across the intestinal epithelium (Knipp, Ho, Barsuhn, & Borchardt, 1997).
- Studying the toxicity of drugs and food metabolites in the intestinal mucosa (Tang, Chikhale, Shah, & Borchardt, 1993).

- To study how components of a formulation i.e. adjuvants or food matrices may affect intestinal epithelial transport of bioactive molecules (Nerurkar, Burton, & Borchardt, 1996).
- Studying the importance of efflux systems in the intestinal epithelium (Burton, Goodwin, Conradi, Ho, & Hilgers, 1997).
- Studying the interactions of bioactive molecules transportation across the intestinal (Wacher, Salphati, & Benet, 1996).

1.6.2.1.4 Advantages and disadvantages of Caco-2

Even though, the Caco-2 cells line has similar characteristics of mature small intestinal enterocytes when differentiated; they have several limitations comparing with normal intestinal epithelium (Lea, 2015a). Firstly, normal intestinal epithelium has more than one type of cells and does not have only enterocytes (Lea, 2015a). Secondly, under normal growth conditions Caco-2 cells do not produce mucins and unstirred water layer is present (Pan, Han, Zhang, Yu, & Liu, 2015).

1.6.2.2 HT29 cell line

In 1964, Fogh and Trempe isolated human colon adenocarcinoma cell line HT29 from a 44 years old Caucasian female (Fogh & Trempe, 1975). The purpose of deriving this cell line was to study the biology of human cancers, but due to their ability to express characteristics of mature intestinal cell like enterocytes or mucus producing cells has attracted attention (Martínez-Maqueda, Miralles, & Recio, 2015).

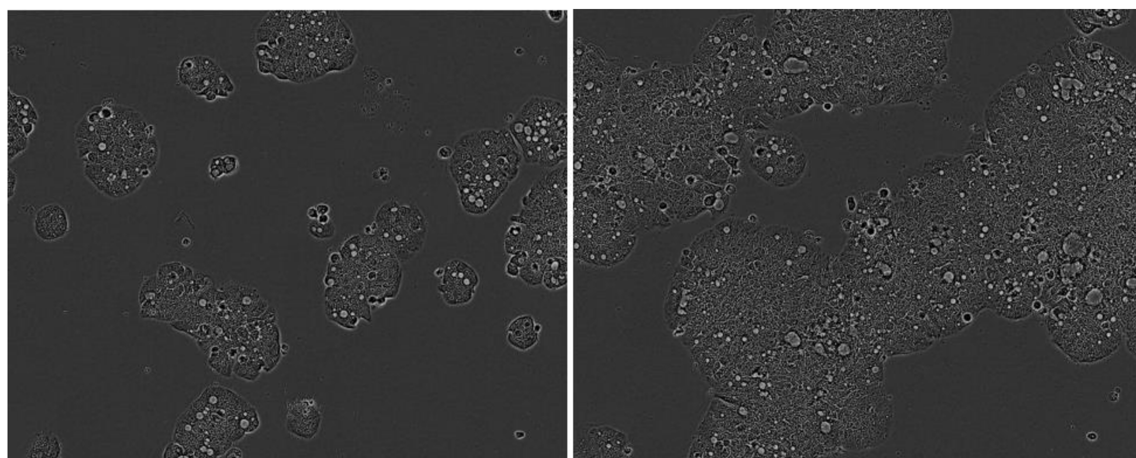

Figure 10: HT29-MTX cells in culture at 72 and 120 hours (PHE, 2021)

1.6.2.2.1 Features and Mechanisms

Glucose consumption in HT29 cell line is high; therefore, culturing them requires a media with high glucose concentration (Martínez-Maqueda et al., 2015). These cells have shown undifferentiated phenotype, as "they grow as a multilayer of unpolarised undifferentiated cells, and functionally they do not express any typical characteristics of intestinal epithelial cells, and they express low amounts of hydrolases" (Martínez-Maqueda et al., 2015). However, replacement of glucose by galactose in the culture medium shows a reversible enterocytic differentiation (Pinto, 1983). When HT29 cells are cultured under appropriate conditions or treated with different inducers, like butyrate (Augeron & Laboisse, 1984) or acid (Fitzgerald, Omary, & Triadafilopoulos, 1997), they become a unique model to study molecular mechanisms of intestinal cell differentiation. Also, they can be modulated to express different pathways of enterocyte differentiation (Martínez-Maqueda et al., 2015). Biochemical markers are used to characterise the polarised phenotype morphologically and physiologically (Martínez-Maqueda et al., 2015).

HT29 cells differentiation is growth-related (Martínez-Maqueda et al., 2015). They form a monolayer with tight junctions between the adjacent cells and apical brush border. Intestinal proteins that normally exist in the intestinal microvilli, such as villin, are also present in the brush border of these differentiated cells (Martínez-Maqueda et al., 2015). Even though, these cells do not express lactase, they express brush border-associated hydrolases that are normally present in intestine, the enzymatic activities of these brush border microvilli are low compared to the *in vivo* normal intestine (Martínez-Maqueda et al., 2015). Enzyme activities such as sucrose-isomaltase, aminopeptidase N, dipeptidylpeptidase-IV and alkaline phosphatase are reached 30 days after culture (Martínez-Maqueda et al., 2015). Enzymes like lactase, are absent in HT29 and the expression of sucrose-isomaltase is between 40-50% (Zweibaum 1986; Zweibaum et al. 2011). However, the production of mucin in the HT29 cells is one of the main differences between HT29 cells and Caco-2 cells (Huet, Sahuquillo-Merino, Coudrier, & Louvard, 1987; Maoret et al., 1989). Exposing HT29 cells to high

concentrations of methotrexate (MTX) up to (10^{-5}mol), it transfers the cells into mucus-secreting differentiated cells (figure 11) (Lesuffleur, Barbat, Dussaulx, & Zweibaum, 1990).

Figure 11: An Insert cross-section of HT29-MTX cells in culture after 21 days, stained with 1% Alcian Blue to highlight the production of mucins (black arrows) (Strugari, Stan, Gharbia, Hermenean, & Dinischiotu, 2018).

As occurs under other metabolic stress conditions like glucose-deprivation, after a high rate of mortality, the resistance to MTX is associated with the cells possessing this stable differentiated mucus-secreting phenotype. Interestingly, cells adapted to low-dose MTX consist of a double population of columnar absorptive and mucus-secreting cells and, at a higher dose, cells are almost exclusively of mucus-secreting phenotype (Martínez-Maqueda et al., 2015). However, when cells are dosed with low amount of MTX the population of columnar absorptive and mucus-secreting cells is double, in contrast with high amount of MTX cells have mucus-secreting phenotype (Martínez-Maqueda et al., 2015). This mucus-secreting phenotype (HT29-MTX) has been used in the transport studies of different compounds, to study the mucus-inducing properties of different food compounds or in studies regarding microorganism’s adhesion and survival (Martínez-Maqueda et al., 2015). It is been reported that tumour-derived cells lack cultivate stability and reproducibility, due to irregularity of growth and non-specific genetic alterations (Lipps, May, Hauser, & Wirth, 2013).

1.6.2.2.2 Relevance to Human *In Vivo* Situation

The gene expression of transporters and metabolic enzymes between normal human intestinal cells and cancer-derived cell line models are significant, which could affect the appropriateness of these models to mimic the *in vivo* permeability (Langerholc et al. 2011). A Comparison of 377 genes expression in HT29 and other epithelium models of intestinal cell lines that are used *in vitro* with the corresponding tissue biopsy, showed no significant differences between differentiated HT29 cells and human colonic tissues (Bourgine et al., 2012).

1.6.2.2.3 Advantages and disadvantages of HT29

Even though, HT29 cell line is used as *in vitro* model of intestinal cells, it has its advantages and limitations. When HT29 cells differentiate, its phenotype is like small intestine enterocytes (Laburthe, Grasset, Louvard, & Zweibaum, 2011; Zweibaum, 1986). Brush border-associated hydrolases presence is associated to the time of differentiation process that could be compared to the one found in the small intestine (Laburthe et al., 2011; Martínez-Maqueda et al., 2015). Also, the expression of villin in differentiation HT29 cells, is close to the value stated for normal freshly prepared colonocytes (Laburthe et al., 2011; Martínez-Maqueda et al., 2015). Despite the advantages, HT29 cells have number of limitations. First, their malignant origin which consume a high rate of glucose and its impairment of the metabolism of glucose. Second, although these cells mimic characteristics of small intestine enterocytes, they are colonic cells. Third, enterocytes in HT29 cells cannot be compared with the ones from normal colon as they express brush border-associated hydrolases, and it cannot be compared with absorptive enterocytes because not all hydrolases are present such as lactase and maltase-glucoamylase (Laburthe et al., 2011; Martínez-Maqueda et al., 2015). It been suggested that HT29 cells are close to human foetal colonic cells because of the type of hydrolases present and the intracellular concentration of glycogen accumulated (Hekmati, Polak-Charcon, & Ben-Shaul, 1990).

In regard of cell surface receptors, it has been reported that there are several receptors that are expressed in differentiated HT29 cells. There are receptors for peptides such as vasoactive and non-peptide substances. Generally, HT29 cells have receptors equivalent to the one found in the normal intestinal cells except for receptor to neurotensin which has be characterised in HT29 cells but it is not detectable in normal human colonic epithelium (Kitabgi et al., 1980). Whereas, normal small intestine epithelial cells have receptors for peptide YY or neuropeptide Y, these receptors are absent in HT29 cells since their origin is the colon (Martínez-Maqueda et al., 2015). It has been reported that some receptors such as opioid, serotonin, muscarinic, and PPARβ/δ are found in this cell line (Ataee, Ajdary, Zarrindast, Rezayat, & Hayatbakhsh, 2010; Belo et al., 2011; Foreman et al., 2011; Zoghbi et al., 2006).

1.7 Bacterial 16S Ribosomal RNA

Ribosomes in living cells are composed of two subunits, one large, and one small, in which an RNA molecule is present. In case of prokaryotic cells, it is identified as 16S, and in eukaryotic cells it is known as 18S. Bacterial 16S rRNA has around 1500 nucleotides which varies between species (Bouchet, Huot, & Goldstein, 2008). Across bacterial groups stretches of this gene, there are conserved areas in the hypervariable regions, where nine of these regions have been recognised known as V1 to V9 (Sarangi et al., 2019) as it shows in (Figure 12).

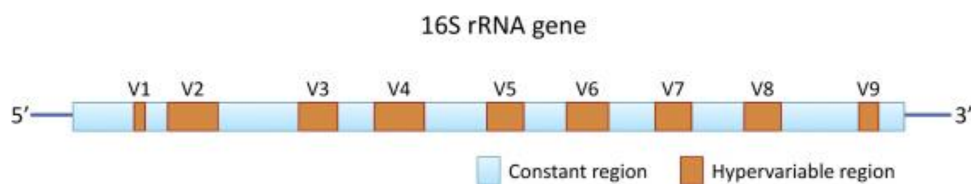

Figure 12: The hypervariable regions (Sarangi et al., 2019).

These variations in the nucleotide sequences reflect on evolutionary divergence of bacteria which provide a reliable method to identify the phylogenetic classification of bacterial species. Identifying bacteria by using nucleotide sequences in these regions has an

advantage that requires no previous bacterial culture. This methodology allows for difficult to culture bacteria to be identified as well as culturable bacteria. Applying these methods to bacterial mixtures provides a reliable assessment on the abundance of different bacterial classes despite their ability to grow in culture (Sarangi et al., 2019).

Bacterial nucleic acid is extracted from the sample, followed by either amplifying the entire length of the 16S rRNA gene or a part of the gene which includes one or more selected hypervariable regions. This methodology uses Polymerase Chain Reaction (PCR) amplification and universal primers corresponding to the preserved regions in the bacterial genome near the entire 16S rRNA or its selected hypervariable regions. The amplified mixture can then be resolved by electrophoresis separation or Fluorescence *In Situ* Hybridization flow cytometry (FISH-flow) and bacterial DNA microarrays (Sarangi et al., 2019; M. C. Thomas, Thomas, Kalmokoff, Brooks, & Selinger, 2012). Because of limited resolution of the above mentioned techniques, newer-generation sequencing methods have been introduced (Sarangi et al., 2019).

1.8 Aim of this project

The interest in studying the effect of the microbiota on the gut is a growing area as it has many implications in human health. Animal models such as germ-free mice have been used to study this interaction but the extrapolation to human health has proven very difficult (Savage, Siegel, Snellen, & Whitt, 1981), hence the need of a reliable *in vitro* model. The intestinal epithelial has been studied in pharmacology for drug safety, drug transport and some drug metabolism, but generally food and nutrients as subject of studies have been left out. Several 2-dimensional (2D) models have been used employing Caco-2 with/without HT29 (intestinal and mucosal cell lines, highly validated), but do not allow proper polarisation (Calatayud, Vázquez, Devesa, & Vélez, 2012; Ghaisas et al., 2016; Kell & Oliver, 2014; Miller et al., 2009; Parr et al., 2016).

3D cell models have emerged as a response to the 3Rs principle (replacement, refine, reduce) and are in majority of the cases focusing on one type of epithelial cells (Caco2) and used for dug assessment. Culturing 3D models requires incubation of the cells (alone or together) in transwell (specialised filter support) in where the cells can be polarised once they reach maturity and differentiation, generally after 21 days (N. Li et al., 2013).

In our novel project, we have set the foundation for the development of a 3D-cell-bacteria model. This co-culture of gut cell (Caco-2 and HT29) has been exposed in the apical side (equivalent of the lumen in the intestine) to different chemicals in the presence and absence of Health promoting bacteria (*Lactobacillus*) and the absorption has been monitored using mass spectrometry methods.

Chapter 2: Materials and Methods

2.1 Materials

All chemical and materials were purchased from Fisher Scientific. Gibco™ Fetal Bovine Serum, Gibco™ DMEM, high glucose, pyruvate, no glutamine, Gibco™ MEM Non-Essential Amino Acids Solution, Gibco™ Trypsin-EDTA (0.25%) Phenol red, Alfa Aesar™ PBS (Phosphate-buffered Saline), Methanol, Optima™ LC/MS Grade (Fisher Chemical), and Lucifer yellow CH, Dilithium salt (≥99%, >95%, MP Biomedicals).

2.2 Cell culture, chemical treatments, and constructs

Cancer ***co****li*-2 (Caco-2) and HT29-MTX were purchased from European Collection of Authenticated Cell Cultures (ECACC). They were regularly maintained in Gibco™ DMEM, high glucose, supplemented with 100 ml of Foetal Bovine Serum (FBS), 5 ml of non-essential amino acids, 5 ml of Glutamine, and 5 ml of antibiotic streptomycin/penicillin. To final concentration 20% FBS, 1% NEAA, 2mM Glutamine, and 100 U/ml of penicillin and 100 ug/ml of streptomycin/penicillin. At 37 °C under an atmosphere of 5% CO2 with 90% relative humidity. The cells were grown in T75 flaks to 70% confluence, and sub-cultured to add the

seeding density through trypsinisation using Gibco™ Trypsin-EDTA (0.25%) 2-4 ml per T75 flask incubated for 3-5 minute to resuspended in new medium.

2.3 Seeding cells in plates

The cells were cultured (Caco-2 passage 43-49, HT29-MTX passage 54-60) in 12-well Corning™ Transwell™ plates (Fisher Scientific) supported with permeable inserts for 21 days to reach maturity and differentiation (Lea, 2015a). Three plates in total were seeded for each cell lines and the co-culturing of both cells at a final density of 3×10^5 cells/cm^2 (Kleiveland, 2015), where the apical side volume is 500µL and the basolateral side volume is 1500µL. The co-cultivation of Caco-2 and HT29-MTX was between 75:25 (Kleiveland, 2015). The cells were maintained with the same culture medium used previously.

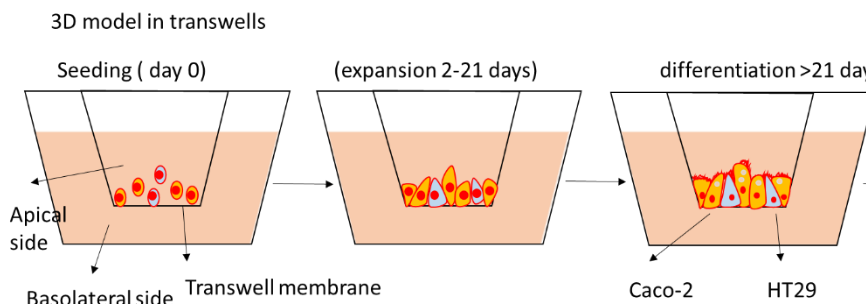

Figure 13: The figure illustrates the way Ccao-2 and HT29-MTX were cultured in the Transwell ™.

2.4 Culturing lactobacillus bacteria

Lactobacillus was cultured in MRS broth (de man, Rogosa, Sharpe) for 24 hours in at 37°C in a shaking incubator (Colombo, Oliveira, Carvalho, & Nero, 2014). The bacteria were provided from Holland & Barrett Acidophilus plus capsules containing *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, and *Lactobacillus casei*.

2.5 Drugs

Erythromycin (E), vitamin D3 (VD3), and linoleic acid (LA) were used to validate the model. Each drug was dissolved in DMSO to make stocks of 10mM.

2.6 Transport Study (Apical → Basolateral)

The flux of the test drug from the apical to the basolateral side was evaluated to predict the absorption of compounds from the lumen of the intestine in the presence of bacteria and mucus. Since this is a High-Throughput Screening protocol, only a single concentration of the test drug and a single incubation time was used. On day 20 Transepithelial/transendothelial electrical resistance (TEER) was taking to verify the integrity of the cellular layer.

2.6.1 Transepithelial Electrical Resistance

There are number of qualitative and quantitative techniques that have been developed to confirm the barrier integrity of endothelial and epithelial cells before proceeding with drug testing, in order to perform reliable in vitro experiments (Srinivasan et al., 2015). Such as, freeze-fracture electron microscopy (Srinivasan et al., 2015), sucrose labelled with carbon-14 for flux measurement on a brain endothelial monolayer (Bowman, Ennis, Rarey, Lorris Betz, & Goldstein, 1983), and radiolabelled markers (Srinivasan et al., 2015). However, these techniques can affect the barrier integrity and interfere with the transport process (Srinivasan et al., 2015). Therefore, Transepithelial/transendothelial electrical resistance (TEER) has been developed to monitor the barrier integrity of cellular monolayers (Srinivasan et al., 2015).

"Transepithelial/transendothelial electrical resistance (TEER) is a very sensitive and reliable method to confirm the integrity and permeability of the monolayer, while the measurement of TEER and trans-epithelial passage of marker molecules are both indicators of the integrity of the tight junctions and of the cell monolayer their experimental parameters are different (Srinivasan et al., 2015; Zucco et al., 2005). As TEER reflects the ionic conductance of the paracellular pathway in the epithelial monolayer, whereas the flux of non-electrolyte tracers (expressed as permeability coefficient) indicates the paracellular water flow, as well as the pore size of the tight junctions (Srinivasan et al., 2015; Zucco et al., 2005)". TEER

method is non-invasive and it can be used to monitor live cells during their various stages of growth and differentiation this give it many advantages (Srinivasan et al., 2015).

As mentioned previously, TEER is used to study the integrity of the barriers formed in the cellular monolayer (Lea, 2015b). The electrodes are placed as one section touching the basolateral chamber and the other section touching the apical chamber. The resistance is measured directly by a portable voltmeter. There are number of equipment to perform the procedures such as Millicell-ERS Voltmeter (Millipore) or the EVOM2, Epithelial Voltohmmeter for TEER (World Precision Instruments Inc., Sarasota, FL).

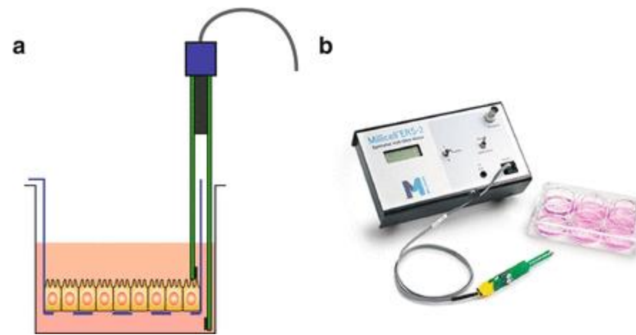

Figure 14: The figure illustrates (a), the electrode when is placed in the chamber. Where the shorter tip of the rod should not contact the cell layer (apical) and the long rod should touch the bottom of the outer chamber (basolateral). (b) the Millicell ERS-2 device with an STX chopstick electrode (Lea, 2015b).

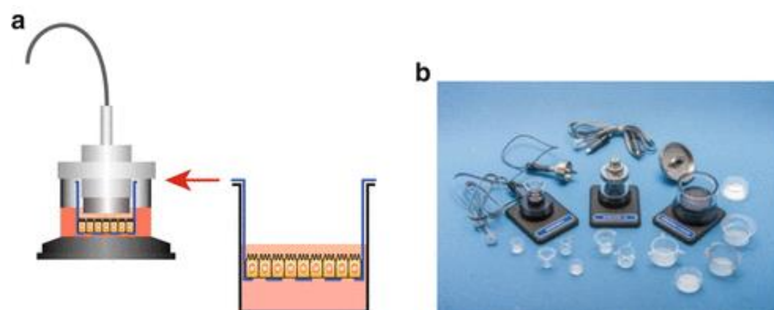

Figure 15: The figure illustrates (a) a schematic drawing of the chamber with culture insert. The gap between the upper rod and the cell layer should be adjusted to 1–2 mm. (b) shows the different devices of the Endohm champers with culture inserts for 6, 12 and 24 well plate inserts (Lea, 2015b).

TEER measurements can be done either by using Ohm's Law Method which has been used in this project or by Impedance Spectroscopy (Srinivasan et al., 2015). Theoretically, ohmic resistance can be determined when a direct current (DC) voltage is applied to the rods, which measure the resulting current (Srinivasan et al., 2015). Then Ohm's law is used to

calculate the ohmic resistance as a ratio between the voltage and current. However, direct current can damage both the cells and the electrodes. Therefore, alternating current (AC) voltage is used with square waveform to overcome this issue (Srinivasan et al., 2015).

2.6.1.1 General protocol for TEER

Depending on the equipment to be used the procedures may differ (Lea, 2015b). If a Millicell STX device is used; First, a blank of the same culture medium is measured by placing the electrode into culture cup without any cells (Lea, 2015b). The shorter rod is placed into the apical side (upper compartment) and the longer rod with be immersed to the basolateral side (lower compartment) as it shows in (Figure 14). The shorter rod should not touch the cell layer. The electrode needs to be held steadily and at 90° angle to the plate insert (Lea, 2015b; Srinivasan et al., 2015).

This method is one of the most common way to measure TEER. However, the readings with the STX electrodes may vary across the cell layer. An alternative method is used to measure the TEER by using Endohm electrode system, which has been developed for EVOM2 Epithelial Voltohmmeter (Figure 15). The resistance is measured by covering the whole area of cells. This is done by taking out the culture wells and insert them the Endohm chamber where the cap has a pair of concentric electrodes with a voltage-sensing silver/silver chloride pellet in the centre plus an annular current electrode(Lea, 2015b; Srinivasan et al., 2015).

2.6.1.2 Calculating Transepithelial Resistance

When the Millicell STX electrode is used the resistance should be measured several times at different places of the monolayer as the uniformity of the cell layer is varied. Then the average value is calculated by subtracting the blank from resistance reading of the sample as it shows in (figure 16). The value then must be multiplied by the surface area of the culture inserts to correct the value, as different inserts have different surface areas (Lea, 2015b).

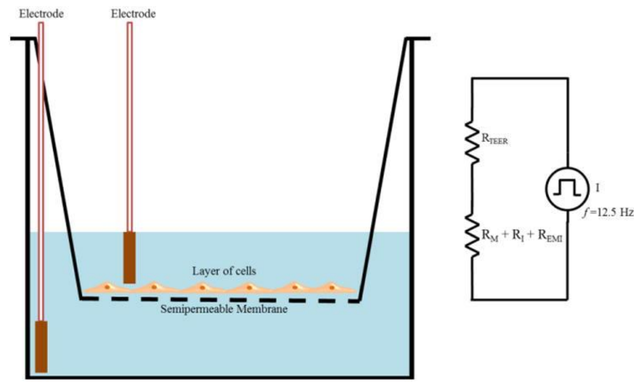

Figure 16: The figure illustrates the way STX electrode is placed in the culture inserts and how the TEER value is calculated (Srinivasan et al., 2015).

2.7 Dosing

On day 21 all the Gibco™ DMEM medium in the plates was removed and replaced with freshly prepared Gibco™ DMEM medium at concentration of 20% FBS, 1% NEAA, 2mM Glutamine subtracting streptomycin/penicillin. Then, 50µL of *Lactobacillus* bacteria to a final concentration of 15 x 10^6 CFU/well (at OD 0.5 at 600 nm) were added to the wells highlighted in blue (figure 17, wells 3 and 4) for all the three plates and incubated for 60 minutes.

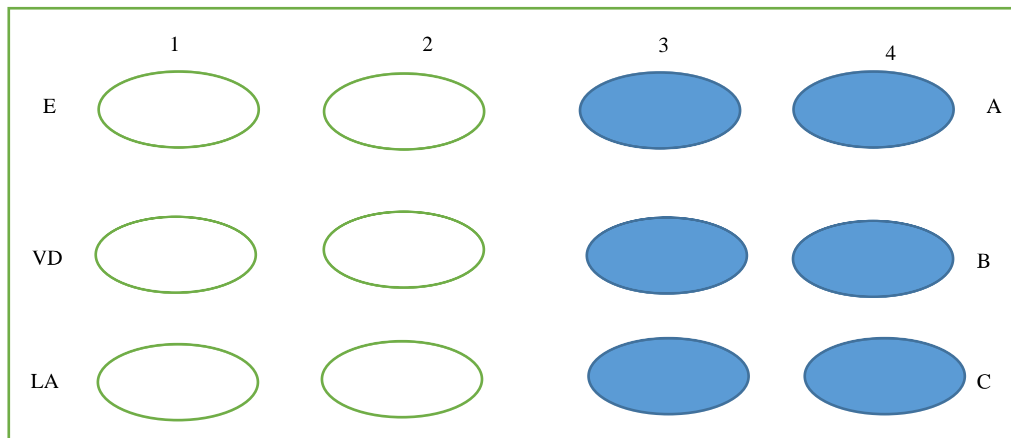

Figure 17: The figure illustrates the plate layout where the drugs were dosed and the bacteria.

Prior dosing the drugs, a stock of 1mM for each drug was made with 100uL of the 10mM stock diluted in 900uL Gibco™ DMEM medium without streptomycin/penicillin. Then 5µL of the drugs from the stock at 1mM were added to all the plates to a finale concentration of 10µM except erythromycin was dosed with 10µL, which brings to a final

concentration of 20μM. At the same time, a 25μL of Lucifer yellow made in Gibco™ DMEM medium without streptomycin/penicillin was dosed in all the wells to test the integrity of the monolayer formation at final concentration of 5μM, which will be discussed more in the next chapter. All the plates were incubated for 60 minutes.

2.8 Lucifer Yellow

Figure 18: The figure illustrates the chemical structure of Lucifer yellow.

Lucifer yellow is a fluorescent dye introduced in 1978 is widely used in cell biology (Hanani, 2012). It is a small, hydrophilic molecule that moves across the blood-brain barrier via passive paracellular diffusion (Omidi et al., 2003; Sharma, Luhach, & Kulkarni, 2019; Watson et al., 2013). Therefore, it has been used as marker for the formation of proper tight junctions (Sharma et al., 2019). Then, the relative fluorescence can be determined at a given time using wavelengths of 485 nm excitation and 529 nm emissions (Sharma et al., 2019).

In this experiment, Lucifer yellow dosed at a final concentration of 5μM, this was achieved by adding 25μL of a stock concentrated at 0.1mM. After the incubation 200μL was taking from basolateral side from all the wells and placed in 96-well plate for fluorescent absorbance using wavelengths of 485 nm excitation and 529 nm emissions. To calculate the percent of Lucifer yellow passage across the cellular layer an equilibrium dilution was made of 25μL of Lucifer yellow plus 500μL, and 1500μL of Gibco™ DMEM medium without streptomycin/penicillin to be the reference solution. The following equation was used to determine the integrity of the cellular layer:

$$RFU\ (test)/RFU\ (equilibrium)x100$$

2.9 Mass spectrometry

Mass spectrometry is a powerful analytical method used to measure the mass-to-charge ratio of ions from small molecules to proteins and oligoes (Hoffmann, 2007). Joseph John Thomson in 1912 developed the first mass spectrometer, since then mass spectrometers have been progressed rapidly that made them to have wide range of applications in many fields in pharmaceuticals to forensic (Baghel, Singh, Singh, & Sinha, 2017; Hoffmann, 2007). “Mass spectroscopy also helps in quantitative elemental analysis, that is, the intensity of a mass spectra signal is directly proportional to the percentage of corresponding element. It is also a non-invasive tool that permits *in vivo* studies in humans. Recent research has investigated the possible applications of mass spectrometers in biomedical field. It is also used as a sensitive detector for chromatographic techniques like LC–MS, GC–MS and LC/MS/MS. These recent hyphenated technological developments of the technique have significantly improved its applicability in pharmaceutical and biomedical analyses” [ch:06] (Baghel et al., 2017).

Liquid chromatography coupled to tandem mass spectrometry LC–MS/MS has been used over the past years for absorption studies and drug discovery. Due to its sensitivity and ability to quantity drugs that have low molecular weight (Bronsema, Bischoff, & van de Merbel, 2012). Another advantage of using LC–MS/MS is the use of internal standard. Internal standard is defined as “a compound that displays physical and chemical characteristics similar to that of the analyte of interest, but at the same time generates a response that can be distinguished from that of the analyte” (Bronsema et al., 2012). To improve the precision and accuracy of the results internal standards are used when analysing samples with mass spectrometry.

In this project, propranolol was selected as internal standard to use for the preparation of the samples.

2.10 Sample preparation for analysis

To prepare the samples for LC–MS/MS, a 15nM solution of the internal standard in methanol (500 ml) was prepared to quench the samples. 300µL sample from the apical and basolateral was quenched into 1200µL and centrifuged at 3800rpm for 20 minutes. Then 500µL of the supernatant was placed in injection vials. The samples were analysed on Agilent 6530 Accurate Mass Q-TOF coupled to an Agilent Infinity 1260 HPLC with a water/methanol (0.1% formic acid) gradient. Erythromycin and vitamin D were analysed in positive ion mode, linoleic acid in negative ion mode, internal standards were used to monitor the sample quality and the chromatographic method, the column used was a standard C18. The gradients were modified for each compound.

Chapter 3: Results and Discussion

3.1 Integrity of the cells

3.1.1 Transepithelial/transendothelial electrical resistance (TEER):

As it mentioned in chapter 1, Caco-2 cell line is the most widely used *in vitro* model to study the intestinal permeability and has number of disadvantages. Hence, the monoculture of Caco-2 cells does not mimic the complexity of the human intestine. Therefore, Intestinal co-culturing models of different types is favoured depending on the desirable application (Kämpfer et al., 2017; Pan et al., 2015).

However, to simulate the human intestinal *in vitro* accurately, it is significantly important to have the right proportions of the two cell lines to culture (Kleiveland, 2015; Pan et al., 2015). Several studies have suggested that co-culturing Caco-2 and HT29-MTX at a ratio of 90:10 or 75:25 is the best ratio for further investigation, based on the TEER values (García-Rodríguez, Vila, Cortés, Hernández, & Marcos, 2018; Georgantzopoulou et al., 2016; Pan et al., 2015).

Alongside the Transwells ™ for the project, a plate of cells a 12 Transwells ™ was cultured at different ratio of 100:0, 90:10, 75:25, 50:50, 30:70, 0:100 (Caco-2:HT29-MTX) in two replicates for each ratio. The ratio 75:25 was chosen after results obtained using TEER and Lucifer yellow as indicators.

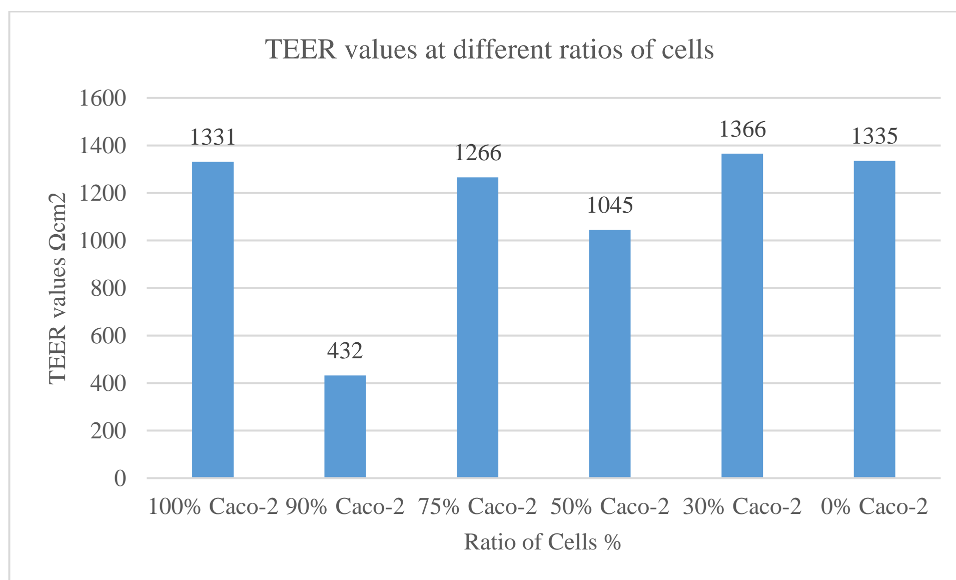

Figure 19: The chart illustrates the average of TEER at different ratios.

As (Figure 19) shows, the average TEER value of Caco-2 cells and HT29-MTX alone was 1331and 1335 Ωcm^2, which was expected. Where the co-culturing wells have an average of 1266 Ωcm^2.

The following chart (Figure 20) shows the TEER values of the Transwells ™ used. The average of TEER values of all the wells in each plate was 1056, 1801, 1026 Ωcm^2, respectively. There is a variation in the average of the four plates. From an experimental point view, TEER values should be higher than 250 Ωcm^2 (Pan et al., 2015), which were achieved.

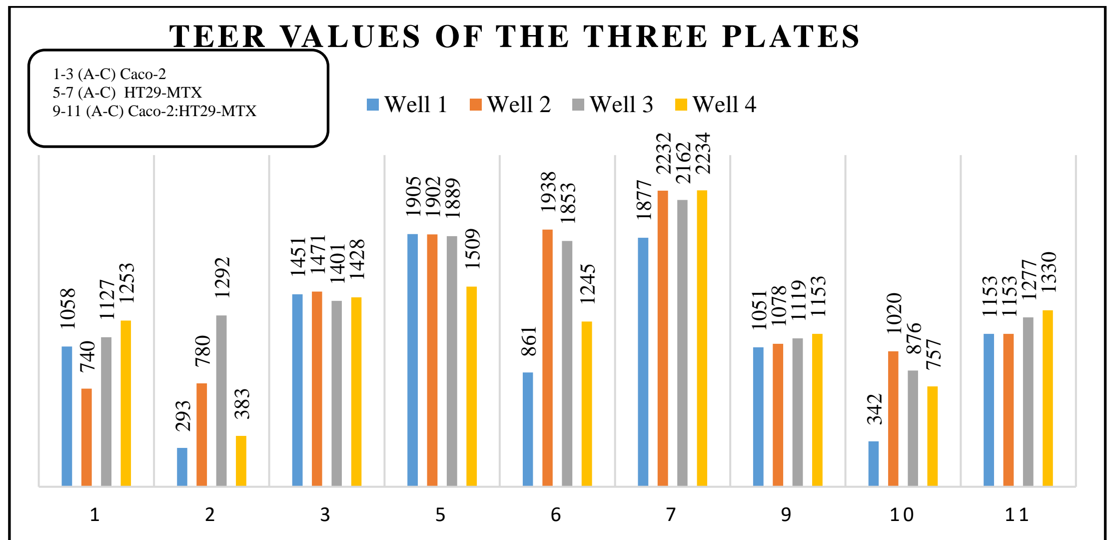

Figure 20: The chart illustrates the TEER readings (Ωcm^2) of Caco-2, HT29-MTX, and Caco-2:HT29-MTX with and without Lactobacillus multiplied by the surface area of the culture inserts 1.13 cm^2.

In the chart (Figure 20), well 1 (B) and well 4 (B) in Caco-2 plate, well 1 (A) in the co-culturing plate had significantly low TEER values comparing to the values of the other wells. This is an indication of something could have happened to the cellular layer formation. Nevertheless, they are still higher than 250 Ωcm^2.

There are number of factors could affect the TEER readings such as, temperature, cell passage number, cell culture medium composition, cell culture period, and shear stress (Srinivasan et al., 2015). However, TEER was not the only method used to test the formation of the tight junctions and the integrity of the monolayer of the cells. Lucifer yellow was used too.

3.1.2 Lucifer Yellow

As it stated in chapter 2, Lucifer yellow is a fluorescent dye used as marker for the formation of proper tight junctions (Sharma et al., 2019). It has been established that the percentage recovery passage of Lucifer yellow should be close to 1% (Cyprotex, 2020). The next chart (Figure 21) shows the percentage recovery passage of Lucifer yellow in the three plates.

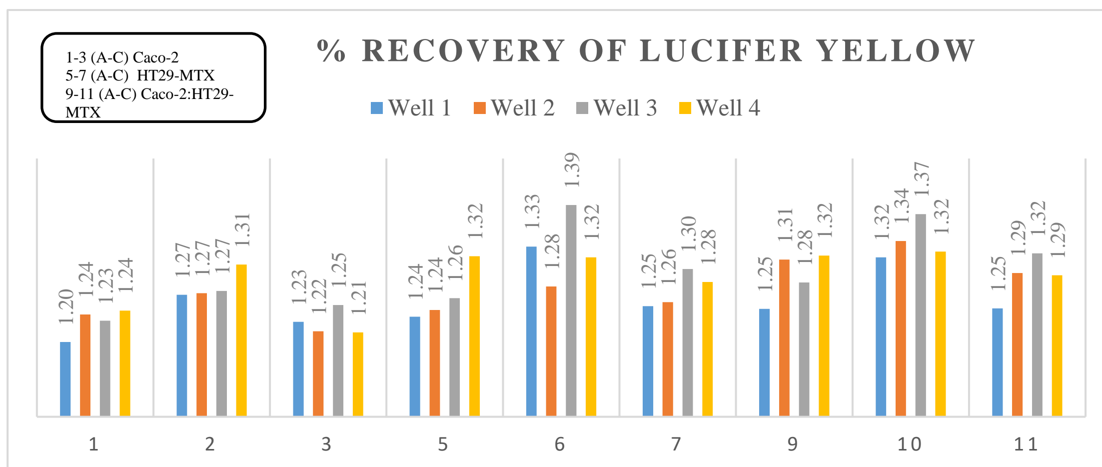

Figure 21: The chart illustrates the percentage recovery passage of Caco-2, HT29-MTX, and Caco-2:HT29-MTX when dosed with the drugs and bacteria.

The recovery percentage of the Lucifer yellow for the three plates from the basolateral side was found to be at 1.28% (±0.04) which is extremely acceptable (Cyprotex, 2020). Though, well 1 (B) and well 4 (B) in Caco-2 plate, well 1 (A) in the co-culturing plate had low TEER values comparing to the other wells the percentage recovery of these wells was still in the acceptable range established. This is an indication that TEER values cannot be the only method to be used to test the integrity of the cellular layer.

The following chart (Figure 22) shows the average percentage recovery of Lucifer yellow when the cells were cultured at different ratios. It illustrates that the ratio 75:25 was ideal to carry out this experiment, as it had an average of 1.09% of Lucifer yellow in the basolateral side.

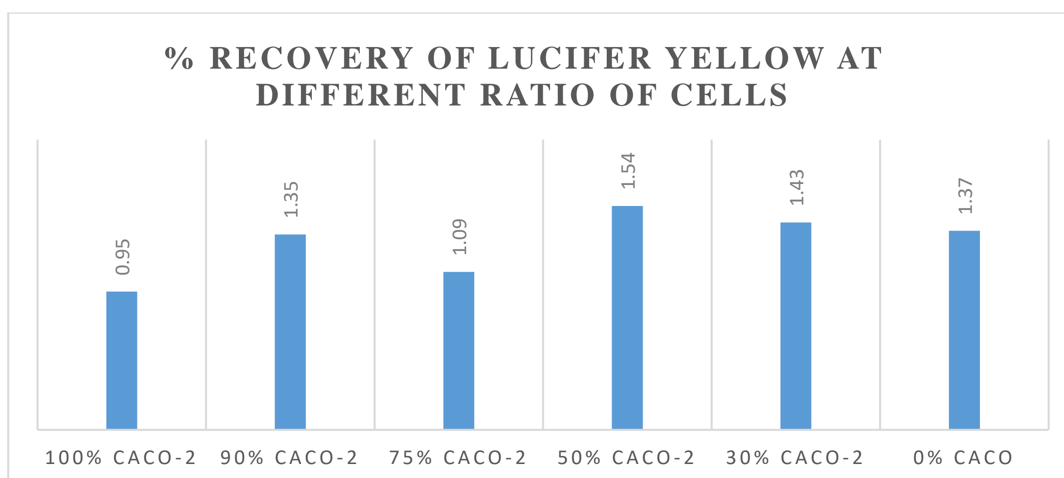

Figure 22: The chart illustrates the percentage recovery passage at different ratios.

It has been mentioned that the cells need at least 21 days to reach maturity on the filter. To confirm this on the following charts (Figure 23 & 24), Caco-2 and HT29-MTX were cultured at different ratios of 150K, 250K, and 350K for 7, 14 and 21 days. TEER and Lucifer yellow were tested at the end of each week.

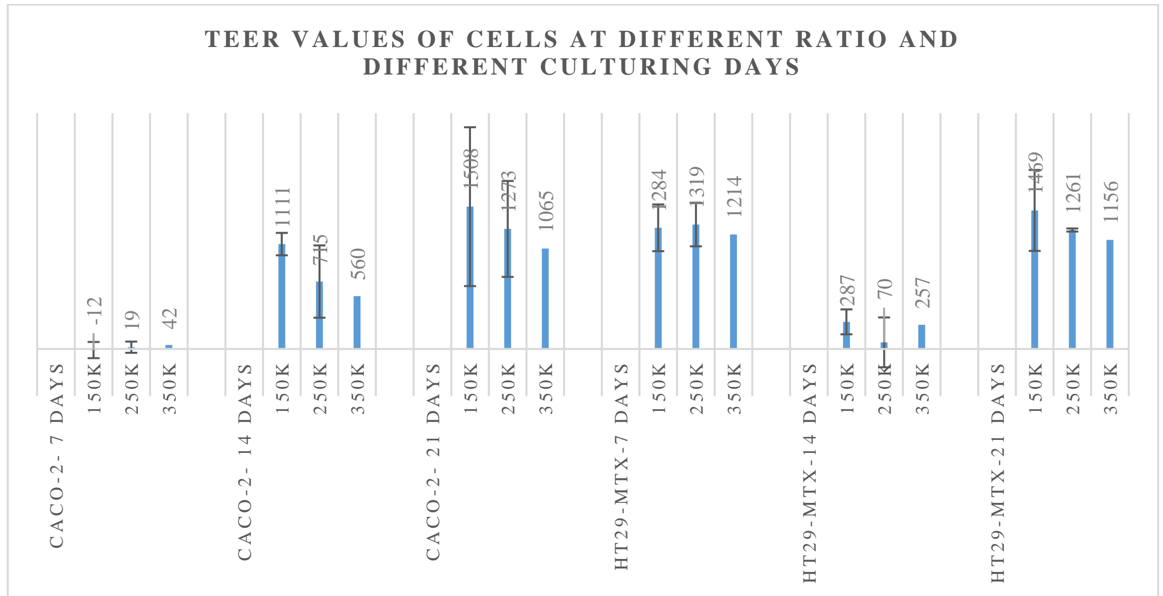

Figure 23: The chart illustrates the average of TEER at different ratio of cells on different days at culturing.

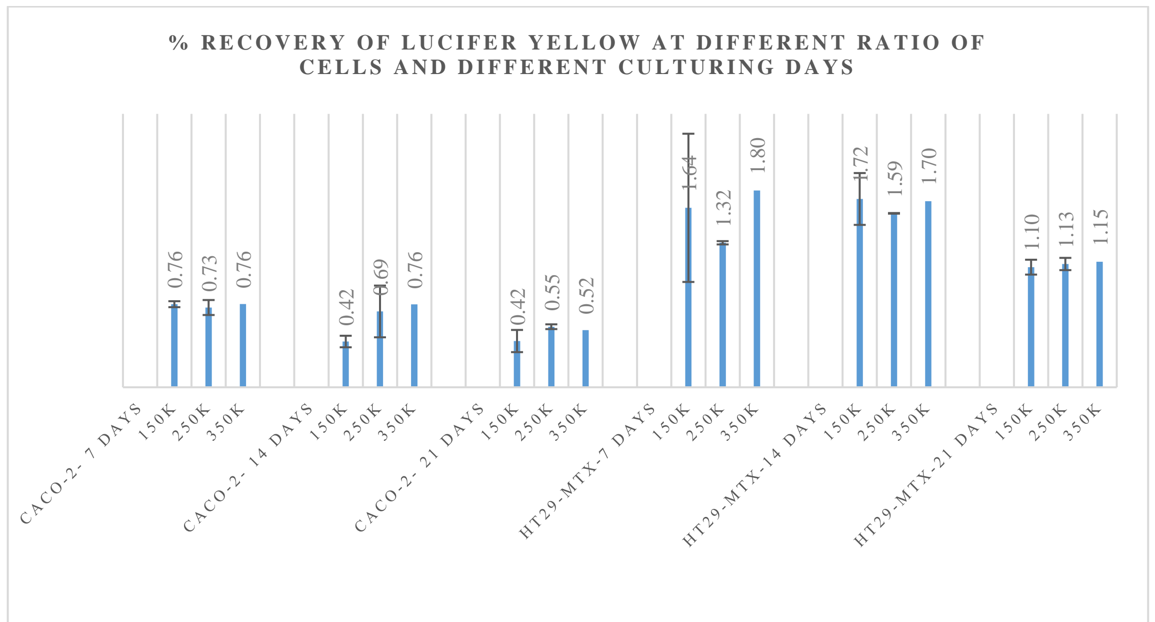

Figure 24: The chart illustrates the percentage recovery passage at different ratio of cells at different days of culturing.

Culturing cells for 21 days provides better TEER values and low percentage recovery passage of Lucifer yellow. As it shows (Figure 23 & 24) both cell lines had over 1000 Ωcm^2 values of TEER and 1% recovery of Lucifer yellow at the end of day 21, which was expected (Cyprotex, 2020; Srinivasan et al., 2015).

3.2 Apparent Permeability Coefficient (Papp):

Apparent permeability coefficient is defined as "the initial flux of compound through the membrane (normalized by membrane surface area and donor concentration) and is typically computed by adapting a straight line to the initial portion of the recorded amounts in the receiver compartment" (Palumbo et al., 2008). This expression is widely used in *in vitro* techniques especially Caco-2 model (Cárdenas et al., 2017), which can determine the rate of any dissolved drugs to pass through the intestinal wall and reaches the systemic circulation (Cárdenas et al., 2017). To calculate Papp the following equation is used:

$$[V.dC/(dT.A.C0)]$$

V sample volume in (ml), dC concentration precents in the sample, A is the surface area of the cellular monolayer in cm^3, and C0 is the donor concentration at time zero. The Papp values are expressed as cm/second. It has been well established in the pharmaceutical industry that $Papp<10^{-6}$ is accounted as poorly permeable while $Papp>10^{-5}$ is permeable (Cyprotex, 2020)

3.4 Erythromycin

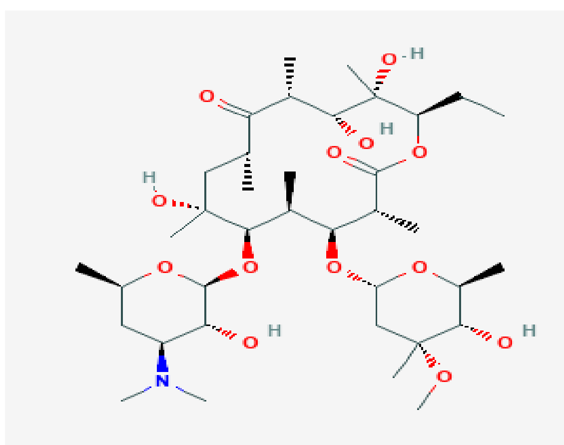

Figure 25: The figure illustrates the chemical structure of erythromycin (PubChem, 2020c).

Erythromycin is a broad-spectrum, macrolide antibiotic. It is used to treat chest infection, skin problems, and sexually transmitted infections (NHS, 2018). It was selected as a control to monitor permeability and tight junction, due to its low permeability across the cellular monolayer (Nožinić, Milić, Mikac, Ralić, & Padovan, 2010). The following graph shows the apparent permeability coefficient (Papp) of erythromycin apical to basolateral.

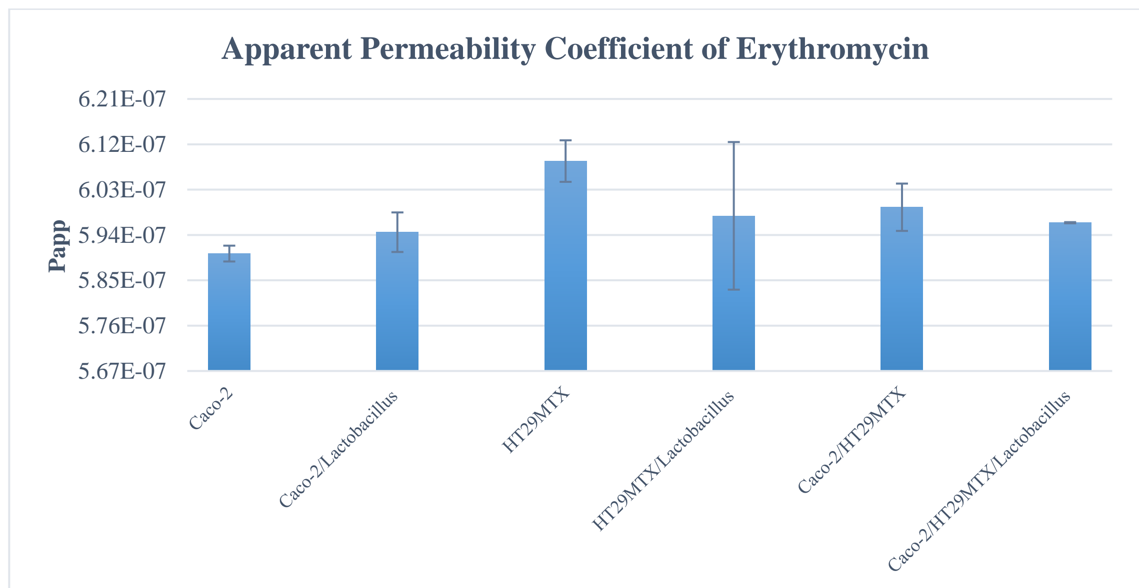

Figure 26: The graph illustrates apparent permeability coefficient of erythromycin.

As expected, the amount found on the basolateral side was minimal providing a Papp in the range of 10^{-7} cm/sec. The lowest Papp value for erythromycin was in Caco-2 followed by Caco-2 with *lactobacillus*, and the combination of the two cell lines with bacteria. Nevertheless, the difference is minimal between all the conditions, and it is possible the mucus produced by HT29-MTX could play a role. This becomes more evident as the highest Papp is for HT29-MTX at 100% (Martínez-Maqueda et al., 2015).

3.5 Vitamin D3

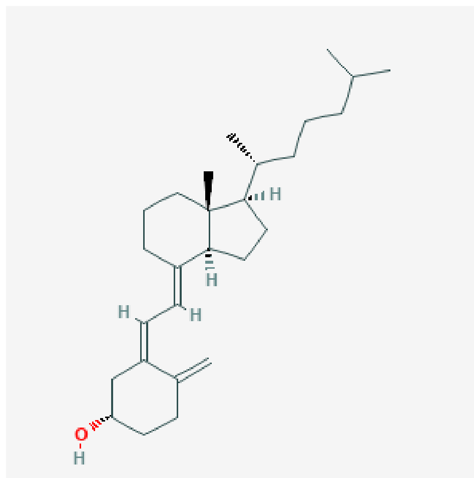

Figure 27: The figure illustrates the chemical structure of Cholecalciferol (PubChem, 2020a).

Vitamin D3 or cholecalciferol is a steroid hormone which can be produced in the skin when exposed to ultraviolet light or acquired from food. The active form of cholecalciferol is 1,25-dihydroxycholecalciferol (calcitriol), that plays an important role to maintain calcium and phosphorus levels in the blood. In addition, its role can mineralise bone. When the active form of vitamin D3 (calcitriol) binds to vitamin D receptors and modulate gene expression this leads to increase the concentrations of calcium. This caused when the intestinal absorption of phosphorus and calcium is increased which promote distal renal tubular to reabsorb calcium and increase osteoclastic resorption (PubChem, 2020a).

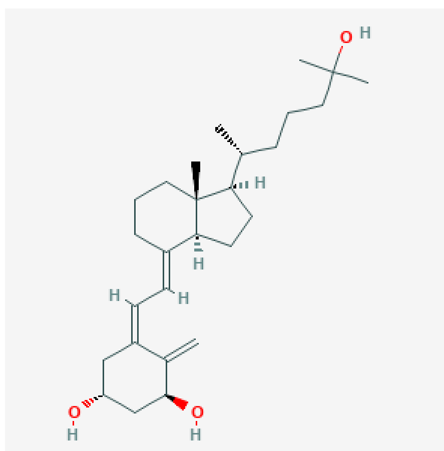

Figure 28: The figure illustrates the chemical structure of calcitriol the active form of cholecalciferol.

Vitamin D3 was not found in the basolateral chamber, therefore the Papp can be considered zero. However, vitamin D3 remained in the apical side as shown in (Table 2). Vitamin D3 is converted in the liver to 25-hydroxyvitamin D by cytochrome P450 2R1

(CYP2R1) and in the kidneys by CYP27B1 to 1,25-dihydroxycholecalciferol (calcitriol) (Ross et al., 2011). The enzymes responsible for these conversions are not present in Caco-2 nor HT29-MTX (Lea, 2015a; Martínez-Maqueda et al., 2015).

Table 2: the concentration of Vitamin D3 in the apical side.

	VD µM remaining
Caco-2	8.6 (6.1)*
Caco-2/bacteria	10.9 (0.3)
HT29-MTX	10.6 (0.7)
HT29-MTX/bacteria	11.2 (1.8)
Caco-2/HT29-MTX	7.8 (1.0)
Caco-2/HT29-MTX /bacteria	13.0 (0.5)

*: standard deviation of two replicates

3.6 Linoleic acid

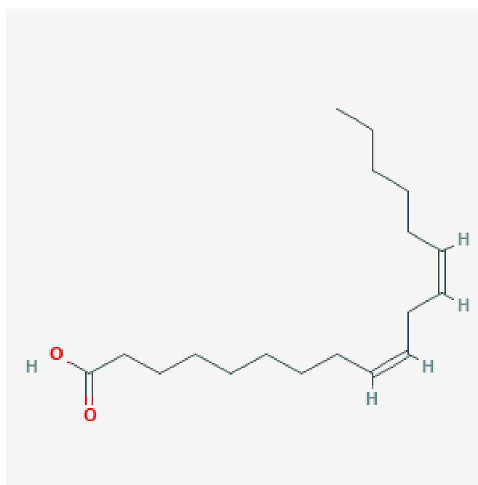

Figure 29: The figure illustrates the chemical structure of Linoleic acid (PubChem, 2020b)

Linoleic acid is a polyunsaturated essential fatty acid, that can be found in oily plants such as sunflower seeds (Meštrović, 2019) and it is essential to human diet (PubChem, 2020b). Linoleic acid is an octadecadienoic acid, in which the two double bonds are at positions 9 and 12 and have Z (cis) stereochemistry (PubChem, 2020b). It has been documented that linoleic acid (cis-9,cis-12-18:2) is metabolised in the human colon via conjugated linoleic acids (CLA) (mainly cis-9,trans-11-18:2) to vaccenic acid (VA) (trans-11-18:1) (Devillard, McIntosh, Duncan, & Wallace, 2007). These conjugated linoleic acids have

gained attention for their roles as anticarcinogenic and lipid/energy metabolism-modulatory effects (Salsinha, Pimentel, Fontes, Gomes, & Rodríguez-Alcalá, 2018). Linoleic acid would not be expected to be found in the basolateral chamber and ideally neither in the apical chamber, as linoleic acid should be metabolised or uptaken by the cells.

As Shown in (Table 3), the Papp of linoleic acid in the basolateral side range from 10^{-6} to 10^{-8} cm/sec which was expected as linoleic acid should be metabolised by *lactobacillus*. It is important to notice that a tenth of the dose concentration is present in the samples of Caco-2 and Caco-2 + *lactobacillus*, implying LA was either metabolised or uptaken. An even lower amount left remaining was present in the cells containing HT29-MTX; as this cell line produces mucus there is a possibility for LA to be uptaken into the mucus layer.

Table 3: the concentration of linoleic acid in the apical side and the Papp in the basolateral side.

	LA µM remaining	LA Papp
Caco-2	1.09 (0.00)*	2.8x10-6 (1.9 x10^{-7})*
Caco-2/bacteria	1.31 (0.14)	1.4x10-6 (1.9 x10^{-6})
HT29-MTX	0.25 (0.04)	1.4 x10-7 (1.6 x10^{-8})
HT29-MTX/bacteria	0.26 (0.00)	1.2 x10-7 (3.6 x10^{-8})
Caco-2/HT29-MTX	0.19 (0.01)	1.4 x10-7 (3.0 x10^{-8})
Caco-2/HT29-MTX/bacteria	0.18 (0.02)	4.5 x10-7 (2.7 x10^{-7})

*: standard deviation of two replicates

Linoleic acid produced other complications, such as ionising in negative mode; this produced some complications as the internal standard was only present in positive ion mode.

Chapter 4: Conclusion

Even though, Caco-2 model is approved by the FDA and EMA for absorption studies as it mimic the human intestinal epithelial cells morphologically and functionally (Pan et al., 2015). As there is a mucus layer separates the epithelial layer from the luminal (Kleiveland, 2015), which is acts as physical and chemical to the intestine from pathogenic invasion (Herath, Hosie, Bornstein, Franks, & Hill-Yardin, 2020). lacking mucus production in Caco-2 cells results low paracellular permeability (Per Artursson, Palm, & Luthman, 2012), which will negatively impact the functionality of enterocytes (Costa & Ahluwalia, 2019).

Therefore, co-cultivation of Caco-2 and HT29-MTX cells has showed promising permits; To investigate bacterial adhesion and invasion in the transportation of molecules across the intestinal epithelium, as the precents of mucus gives complexity to the model to comply with the *in vivo* situation (Kleiveland, 2015).

Our aim is to replace the Caco-2 model with an upgraded version (MiGut), ensuring the addition of bacteria will not affect the permeability and tight junctions precent in the current model. The data clearly shows this co-culture of Caco-2 cells providing tight junctions and epithelial base and HT29-MTX providing the goblet type of cells and the mucus, alongside commensal bacteria is an appropriate model to emulate the human gut for pharmaceutical and biological studies.

To develop and validate a 3D human gut microbiota a baseline is needed to assess if the addition of HT29-MTX with bacteria will not affect the permeability and tight junctions of the favoured Caco-2 model. Further investigating is needed involving number of stages:

1- Repeating the linoleic acid assessment using a different internal standard and monitor the appearance of metabolites.
2- Monitor the uptake of erythromycin, vitamin D3, and linoleic acid in the cells, mucus and bacteria.
3- Explore other strains of *Lactobacillus*.
4- Explore other molecules for absorption and metabolisation.

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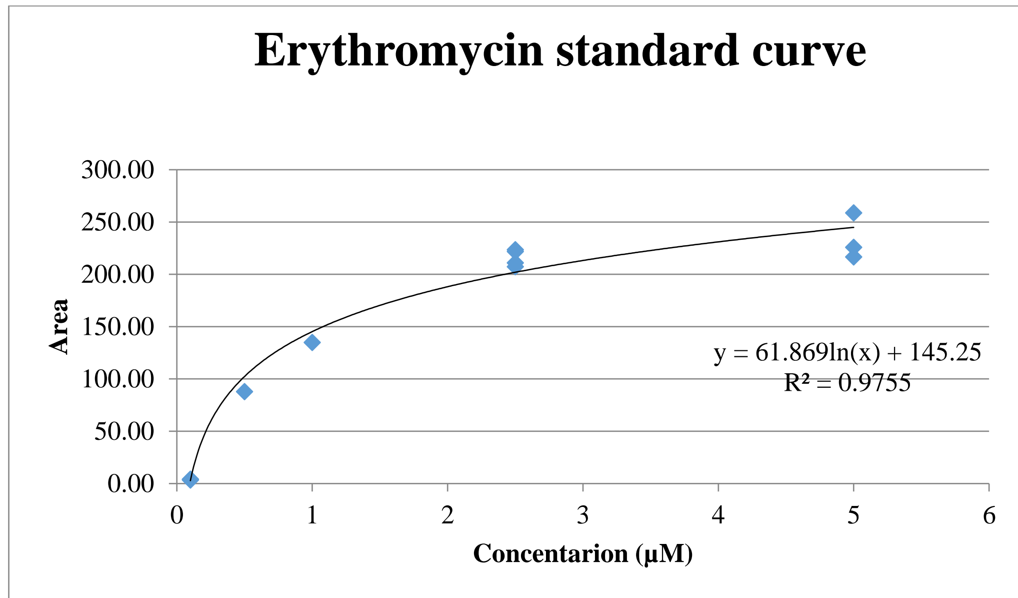
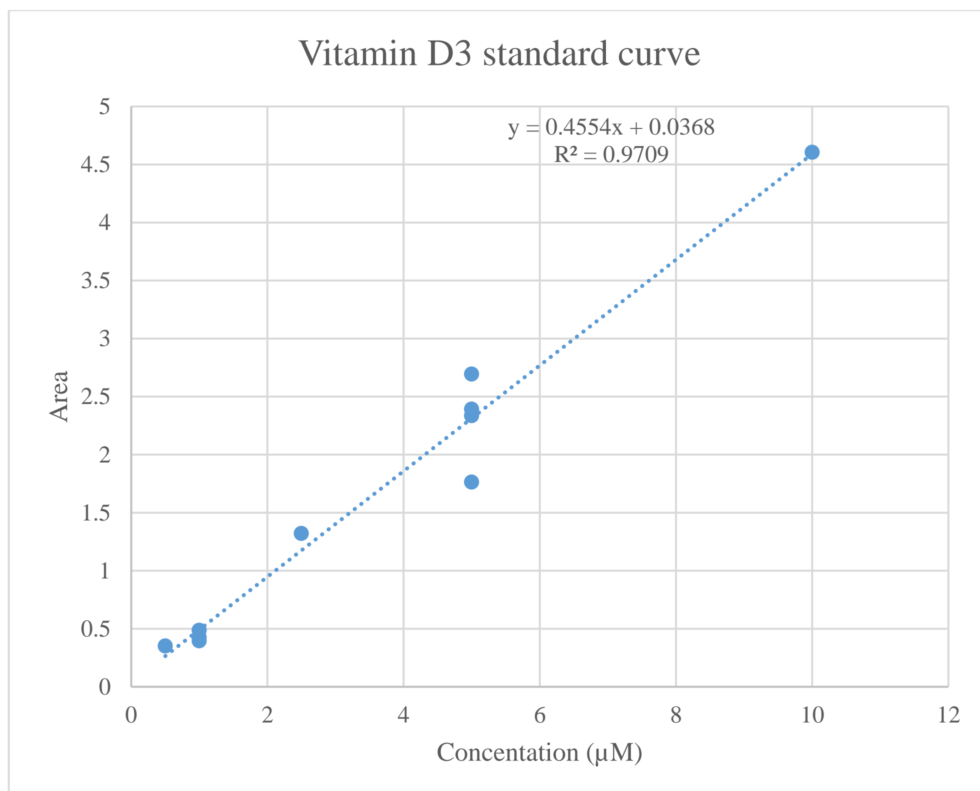
Appendix:

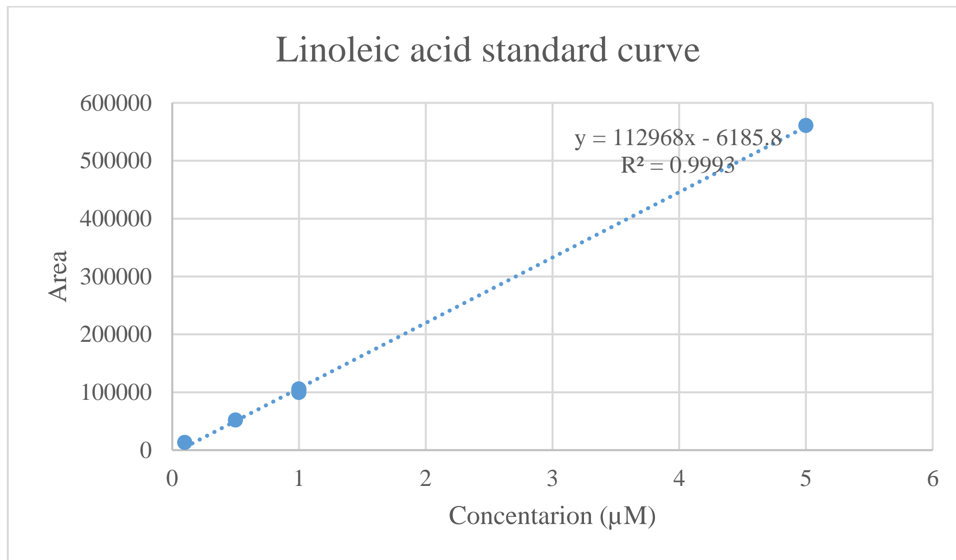
Appendix 1: the stocks for the standard curve

10uM	1.2ul(10mM)+298.8ul MeOH alone	+ 1200ul MeOH with IS
5uM	0.6ul(10mM)+299.4 MeOH alone	+ 1200ul MeOH with IS
1uM	15ul(100uM)+285 MeOH alone	+ 1200ul MeOH with IS
0.5uM	7.5ul(100uM)+292.5 MeOH alone	+ 1200ul MeOH with IS

Appendix 2: the standard curve of the three drugs used.







Appendix 3: the concentration of the samples obtained from mass spectrometry.

	A	B	C	D	E	F	G	H	I	J	K	L	M
1	P1-A10	blank - methanol		Area corrected	Conc (uM)		Papp		Papp	stdev			
2	P1-A11	caco-2 A1 A erythromycin		143.18	0.97	Apical					area	1.13	cm2
3	P1-B1	caco-2 A1 B erythromycin		0.19	0.10	basolateral	5.89E-07	A2B E Caco-2	5.90E-07	1.57E-09	time	3600	sec
4	P1-B2	caco-2 A2 A erythromycin		144.79	0.99	Apical					vol AtoB	1.5	ml basolateral
5	P1-B3	caco-2 A2 B erythromycin		0.42	0.10	basolateral	5.91E-07				vol BtoA	0.5	ml apical
6	P1-B4	caco-2 bact A3 A erythromycin		139.33	0.91	Apical					formula = Papp=(vol basolateral * conc basolateral)/(area*time*20uM)		
7	P1-B5	caco-2 bact A3 B erythromycin		1.03	0.10	basolateral	5.97E-07	A2B E Caco-2 + bact	5.95E-07	3.93E-09			
8	P1-B6	caco-2 bact A4 A erythromycin		146.07	1.01	Apical							
9	P1-B7	caco-2 bact A4 B erythromycin		0.45	0.10	basolateral	5.92E-07						
10	P1-B8	ht29 A1 A erythromycin		136.56	0.87	Apical							
11	P1-B9	ht29 A1 B erythromycin		2.49	0.10	basolateral	6.12E-07	A2B E HT29	6.09E-07	4.12E-09			
12	P1-B10	ht29 A2 A erythromycin		161.36	1.30	Apical							
13	P1-B11	ht29 A2 B erythromycin		1.90	0.10	basolateral	6.06E-07						
14	P1-C1	ht29 bact A3 A erythromycin		141.13	0.94	Apical							
15	P1-C2	ht29 bact A3 B erythromycin		2.14	0.10	basolateral	6.08E-07	A2B E HT29 + bact	5.98E-07	1.47E-08			
16	P1-C3	ht29 bact A4 A erythromycin		0.30	0.10	Apical							
17	P1-C4	ht29 bact A4 B erythromycin			0.10	basolateral	5.87E-07						
18	P1-C5	caco-2+ht29 A1 A erythromycin		136.77	0.87	Apical							
19	P1-C6	caco-2+ht29 A1 B erythromycin		1.60	0.10	basolateral	6.03E-07	A2B E HT29 + caco2	6.00E-07	4.70E-09			
20	P1-C7	caco-2+ht29 A2 A erythromycin		202.13	2.51	Apical							
21	P1-C8	caco-2+ht29 A2 B erythromycin		0.91	0.10	basolateral	5.96E-07						
22	P1-C9	caco-2+ht29 bact A3 A erythromycin		145.41	1.00	Apical							
23	P1-C10	caco-2+ht29 bact A3 B erythromycin		0.93	0.10	basolateral	5.96E-07	A2B E HT29 + caco2 + bact	5.96E-07	1.18E-10			
24	P1-C11	caco-2+ht29 bact A4 A erythromycin		150.59	1.09	Apical							

Sheet3 | Chart1 | Sheet1 | Sheet2

	A	B	C	D	E	F	G	H	I	J	K	L
1	P1-A10	blank - methanol	Area corrected	Conc (uM)		Papp		Papp	stdev			
2	P1-B1	caco-2 C1 Alinoleic			Apical					area	1.13	cm2
3	P1-B1	caco-2 C1 B linoleic	83857.93	0.237	basolateral	2.91E-06	A2B LA Caco-2	2.77E-06	1.93E-07	time	3600	sec
4	P1-B2	caco-2 C2 A linoleic	321813.08	1.086	Apical					vol AtoB	1.5	ml basolateral
5	P1-B3	caco-2 C2 B linoleic	77637.66	0.214	basolateral	2.63E-06	A2B LA Caco-2			vol BtoA	0.5	ml apical
6	P1-B4	caco-2 bact C3 A linoleic	412814.56	1.411	Apical					formula = Papp=(vol basolateral * conc basolateral)/(area*time*10uM)		
7	P1-B5	caco-2 bact C3 B linoleic	80512.30	0.225	basolateral	2.76E-06	A2B LA Caco-2 + bact	1.40E-06	1.92E-06			
8	P1-B6	caco-2 bact C4 A linoleic	358407.62	1.217	Apical							
9	P1-B7	caco-2 bact C4 B linoleic	18701.31	0.004	basolateral	4.85E-08	A2B LA Caco-2 + bact					
10	P1-C5	ht29 C1 A linoleic	78913.67	0.219	Apical							
11	P1-C6	ht29 C1 B linoleic	20953.01	0.012	basolateral	1.47E-07	A2B LA HT29	1.36E-07	1.59E-08			
12	P1-C7	ht29 C2 A linoleic	94340.35	0.274	Apical							
13	P1-C8	ht29 C2 B linoleic	20440.60	0.010	basolateral	1.25E-07	A2B LA HT29					
14	P1-C9	ht29 bact C3 A linoleic	91189.14	0.263	Apical							
15	P1-C10	ht29 bact C3 B linoleic	19696.26	0.007	basolateral	9.22E-08	A2B LA HT29 + bact	1.18E-07	3.59E-08			
16	P1-C11	ht29 bact C4 A linoleic	90143.63	0.259	Apical							
17	P1-D1	ht29 bact C4 B linoleic	20852.66	0.012	basolateral	1.43E-07	A2B LA HT29 + bact					
18	P1-B8	caco-2+ht29 C1 A linoleic	68193.86	0.181	Apical							
19	P1-B9	caco-2+ht29 C1 B linoleic	21168.28	0.013	basolateral	1.57E-07	A2B LA HT29 + caco2	1.35E-07	3.01E-08			
20	P1-B10	caco-2+ht29 C2 A linoleic	71998.59	0.194	Apical							
21	P1-B11	caco-2+ht29 C2 B linoleic	20198.41	0.009	basolateral	1.14E-07	A2B LA HT29 + caco2					
22	P1-C1	caco-2+ht29 bact C3 A linoleic	72218.21	0.195	Apical							
23	P1-C2	caco-2+ht29 bact C3 B linoleic	23542.24	0.021	basolateral	2.61E-07	A2B LA HT29 + caco2 + bact	4.51E-07	2.68E-07			
24	P1-C3	caco-2+ht29 bact C4 A linoleic	65581.98	0.171	Apical							
25	P1-C4	caco-2+ht29 bact C4 B linoleic	32188.96	0.052	basolateral	6.40E-07	A2B LA HT29 + caco2 + bact					

A	B	D	E	F	G	H	I	J	K	L	M
1-A10	blank - methanol	Area corrected	Conc (uM)		Papp		Papp	stdev			
1-A9	caco-2 B1 A vit d	2.02	4.36	Apical					area	1.13	cm2
1-A10	caco-2 B1 B vit d	0.00		basolateral	0.00E+00	A2B E Caco-2	0.00E+00	0.00E+00	time	3600	sec
1-A11	caco-2 B2 A vit d	5.92	12.92	Apical					vol AtoB	1.5	ml basolateral
1-B1	caco-2 B2 B vit d	0.00		basolateral	0.00E+00				vol BtoA	0.5	ml apical
1-B2	caco-2 bact B3 A vit d	4.92	10.72	Apical					formula = Papp=(vol basolateral * conc basolateral)/(area*time*10uM)		
1-B3	caco-2 bact B3 B vit d	0.00		basolateral	0.00E+00	A2B E Caco-2 + bact	0.00E+00	0.00E+00			
1-B4	caco-2 bact B4 A vit d	5.08	11.07	Apical							
1-B5	caco-2 bact B4 B vit d	0.00		basolateral	0.00E+00						
1-C3	ht29 B1 A vit d	4.61	10.03	Apical							
1-C4	ht29 B1 B vit d	0.00		basolateral	0.00E+00	A2B E HT29	0.00E+00	0.00E+00			
1-C5	ht29 B2 A vit d	5.09	11.09	Apical							
1-C6	ht29 B2 B vit d	0.00		basolateral	0.00E+00						
1-C7	ht29 bact B3 A vit d	5.70	12.43	Apical							
1-C8	ht29 bact B3 B vit d	0.00		basolateral	0.00E+00	A2B E HT29 + bact	0.00E+00	0.00E+00			
1-C9	ht29 bact B4 A vit d	4.56	9.92	Apical							
1-C10	ht29 bact B4 B vit d	0.00		basolateral	0.00E+00						
1-B6	caco-2+ht29 B1 A vit d	3.26	7.08	Apical							
1-B7	caco-2+ht29 B1 AB vit d	0.00		basolateral	0.00E+00	A2B E HT29 + caco2	0.00E+00	0.00E+00			
1-B8	caco-2+ht29 B2 A vit d	3.89	8.45	Apical							
1-B9	caco-2+ht29 B2 B vit d	0.00		basolateral	0.00E+00						
1-B10	caco-2+ht29 bact B3 A vit d	5.77	12.60	Apical							
1-B11	caco-2+ht29 bact B3 B vit d	0.00		basolateral	0.00E+00	A2B E HT29 + caco2 + bact	0.00E+00	0.00E+00			
1-C1	caco-2+ht29 bact B4 A vit d	6.11	13.33	Apical							
2	caco-2+ht29 bact B4 B vit d	0.00		basolateral	0.00E+00						

Appendix 4: The fluorescent absorbance of Lucifer yellow

	A	B	C	D	E	F	G	H	I	J	K	L	M
1	1	2	3	4	5	6	7	8	9	10	11		
2	1580	1629	1618	1636	2067	3557	2173	1639	1727	1686	1734		
3	1664	1667	1671	1718	1954	3442	2159	1731	1760	1808	1741		
4	1616	1599	1646	1597	2062		2244	1640	1703	1738	1699		
5	1625	1637	1658	1733	MEDIA	APICAL	EQUILIBRUM						
6	1750	1679	1824	1731	2027.67	3499.5	2192						
7	1644	1651	1710	1687			1186						
8													
9													
10													
11	CACO2												
12	HT29												
13	CACO+HT29												